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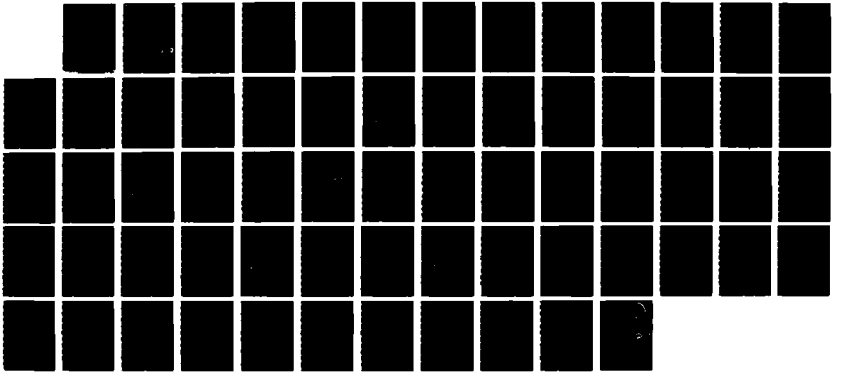

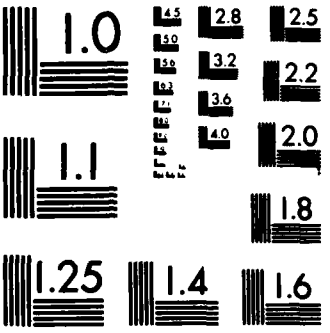

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ALTERATION OF THE EFFICACY AND TOXICITY OF CLINICALLY USEFUL DRUG ESTERS BY ORGANOPHOSPHATE INHIBITORS

Annual Report

Mickey Castle

May 22, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Eastern Virginia Medical School
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The present studies are intended to develop animal models for investigations of the hydrolysis of therapeutically useful drug esters, especially those hydrolyzed by carboxylesterases. These models are to be used to investigate the effects of organophosphate compounds on ester hydrolysis in vitro and in vivo. The results of these investigations will permit more rational and predictable drug therapy for individuals who have been exposed to organophosphate compounds. Liver microsomes were isolated from untreated guinea pigs and subsequently incubated with one of several ester substrates (chloramphenicol succinate, prednisolone succinate, procaine, procaineamide, lidocaine, methylparaben and meperidine). Only four of the substrates exhibited significant hydrolysis. Chloramphenicol succinate and prednisolone succinate hydrolysis were found to have first-order reaction rates and to be inhibited by paraoxon in a manner consistent with coupled or uncompetitive inhibition. Procaine hydrolysis, the most rapid, was inhibited by

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paraoxon in a purely competitive manner. Methylparaben hydrolysis followed zero-order kinetics and the Lineweaver-Burk plot produced negative slopes. In in vivo studies, paraoxon was administered to guinea pigs (15 ug/kg, either 1 hr or 12 hr before sacrifice; 150 ug/kg, either 1 hr or 12 hr before sacrifice). The results of these investigations demonstrate that carboxylesterase activity toward several esters of drugs is present in liver, lung and kidney. The liver is by far the most important site of hydrolysis of these ester compounds. Some substrates are hydrolyzed by the lung and kidney to a greater extent than other substrates. The administration of paraoxon, even in high doses, failed to produce a consistent or pronounced inhibition of carboxylesterase activity. In some cases, pretreatment with paraoxon actually increased enzyme activity. These results suggest that paraoxon pretreatment may cause either inhibition or enhancement of enzyme activity in the guinea pig.

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ABSTRACT

The present studies are intended to develop animal models for investigations of the hydrolysis of therapeutically useful drug esters, especially those hydrolyzed by carboxylesterases. These models were used to investigate the effects of organophosphate compounds on ester hydrolysis *in vitro* and *in vivo*. The results of these investigations will permit more rational and predictable drug therapy for individuals who have been exposed to organophosphate compounds. Liver microsomes were isolated from untreated guinea pigs and subsequently incubated with one of several ester substrates (chloramphenicol succinate, prednisolone succinate, procaine, procaineamide, lidocaine, methylparaben and meperidine). Only four of the substrates exhibited significant hydrolysis: Chloramphenicol succinate and prednisolone succinate hydrolysis were found to have first-order reaction rates and to be inhibited by paraoxon in a manner consistent with coupled or uncompetitive inhibition. Procaine hydrolysis, the most rapid, was inhibited by paraoxon in a purely competitive manner. Methylparaben hydrolysis followed zero-order kinetics and the Lineweaver-Burk plot produced negative slopes. In *in vivo* studies, paraoxon was administered to guinea pigs (15 ug/kg, either 1 hr or 12 hr before sacrifice; 150 ug/kg, either 1 hr or 12 hr before sacrifice). The results of these investigations demonstrate that carboxylesterase activity toward several esters of drugs is present in liver, lung and kidney. The liver is by far the most important site of hydrolysis of these ester compounds. Some substrates are hydrolyzed by the lung and kidney to a greater extent than other substrates. The administration of paraoxon, even in high doses, failed to produce a consistent or pronounced inhibition of carboxylesterase activity. In some cases, pretreatment with paraoxon actually increased enzyme activity. These results suggest that paraoxon pretreatment may cause either inhibition or enhancement of enzyme activity in the guinea pig.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978). Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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INTRODUCTION

Background. A number of studies have suggested that esterase enzymes may be important in the metabolism of certain drugs. The two most important classes of esterases in this regard are amidases and carboxylesterases, although much discussion has centered around whether these enzymes do indeed have different substrate specificities. These enzymes are located in the blood as well as in several tissues, especially the liver, kidney and lung. The specificity of these enzymes appears to be quite thus different from that of acetylcholinesterase (E.C.3.1.1.7.), since the latter enzyme has very little activity toward esters formed from amines or carboxylic acids. Instead, these substrates are hydrolyzed by a group of esterases frequently referred to as carboxylesterases (E.C.1.1.1.).

The authoritative review by Heyman (1) summarizes much of the current knowledge concerning carboxylesterase enzymes. Important points relevant to this discussion include the following: 1) "Carboxylesterase" refers to a large group of enzymes present in a variety of tissues. The amounts of these enzymes in the blood vary from species to species, with low levels in humans and guinea pigs and very high levels in rat. The livers of all species contain large amounts of these enzymes: 2) There are marked differences between carboxylesterases and other esterases, such as acetylcholinesterase, with regard to the extent of inhibition by compounds such as organophosphates: 3) Although a few drug esters have been shown to be substrates for carboxylesterases, most of the substrates identified so far are esters which have no clinical significance.

Rationale. A number of studies have suggested that carboxylesterases may be important in the disposition of certain therapeutically useful drug esters. Several steroid hormone esters are hydrolyzed *in vitro* by pig liver esterases (2). Studies *in vitro* with rat liver microsomes (3) and *in vivo* in mice (4) have shown that procaine is also a substrate for these enzymes. The esterase responsible for the hydrolysis of meperidine in rats was demonstrated to be a microsomal enzyme and to be inhibited by the presence of other esters (5). The hydrolysis of aspirin to salicylic acid is mediated by an esterase in the liver (6).

A variety of other drugs contain ester linkages, the detoxification and elimination processes required to terminate their activity may rely upon hydrolysis. Certain analgesics, antibiotics, anesthetic agents, antiinflammatory agents and cardiovascular agents are included in this group. Inhibition of ester hydrolysis in this group of drugs would lead to an accumulation of the active drug in the body with a concomitant increase in the duration of drug action and toxicity, even with normally therapeutic doses.

A wide variety of drugs have also been synthesized as the prodrug ester which is inactive until it is hydrolyzed in the body to release the active component. In most cases, this formulation is designed to permit normally insoluble drugs to be used for intravenous or intramuscular injections. In other cases, the prodrug serves as a depot to achieve sustained release of the active drug over a period of time. The liberation of the active drug from the ester is frequently dependent upon the activity of esterase enzymes located in the blood or tissues. Inhibition of this esterase activity would thus prevent the prodrug from being converted to the active form. Again drugs such as antibiotics, analgesics and antiinflammatory agents may produce unexpected results in patients exposed to inhibitors of esterase enzymes.

Purpose. The present studies are intended to develop animal models for investigations of the hydrolysis of therapeutically useful drug esters, especially those hydrolyzed by carboxylesterases. These models will also be used to investigate the effect of organophosphates on the hydrolysis of these esters both in vitro and in vivo.

Drug Esters.

1) Lidocaine. This drug is widely used as both a local anesthetic agent and an antiarrhythmic agent. Lidocaine is an amide ester but does not contain a carboxylester linkage. Use of this substrate will provide additional information on whether hydrolysis of amide esters and carboxylesters occurs via a single esterase or multiple esterases. The product of amide hydrolysis of lidocaine is 2,6-dimethylaniline. Ester hydrolysis plays a major role in the inactivation and excretion of lidocaine (7).

2) Chloramphenicol. This broad-spectrum antibiotic, which is insoluble in water, is frequently administered orally. In order to provide a form of the drug which can be administered intravenously, an ester (chloramphenicol succinate) has been synthesized. This esterified form of chloramphenicol is inactive and must be hydrolyzed to liberate active chloramphenicol. This hydrolysis is brought about by carboxylesterases located in the liver, kidney and lung (M.C. Castle, unpublished results). There appears to be very little hydrolysis in the blood. Ester hydrolysis, which is essential for therapeutic activity, is highly variable from one individual to another (8).

3) Prednisolone. This antiinflammatory agent is also available as the succinate ester for intravenous administration. The sites of activation to prednisolone are not known but are probably the same as those for chloramphenicol succinate. Inhibition of hydrolysis would lead to reduced therapeutic activity.

4) Meperidine. An analgesic which belongs to the opioid class of compounds, meperidine is widely used for the control of mild to moderate pain. The major metabolic pathway leading to inactivation of meperidine is hydrolysis of the carboxylester to form meperidinic acid, which is then conjugated and excreted (9) Inhibition of hydrolysis would lead to accumulation of the drug in the body and potential toxic reactions.

5) Procaine and Procaineamide. The primary use of procaine is as a local anesthetic. However, procaine is also a component of other drug preparations such as salts of penicillin used for intramuscular administration. Hydrolysis of the carboxylester is necessary for inactivation and excretion (10). The product of hydrolysis of procaine is p-aminobenzoic acid (PABA). Procaineamide differs from procaine merely by replacement of the ester linkage with an amide linkage. Its primary use is as an antiarrhythmic agent. This is another substrate which should yield information concerning the specificity of esterases present in the microsomal fraction of guinea pig liver. It may be possible to differentiate amidases (i.e., those that hydrolyze procaineamide) from carboxylesterases (i.e., those that hydrolyze procaine).

6) Methylparaben. Many intravenous solutions contain methylparaben as a preservative. The amount of this preservative present in these solutions is not usually large enough to cause problems, in part because the body is able to metabolize and excrete the drug readily. This metabolism involves hydrolysis of the methyl ester by carboxylesterase enzymes (10) to form p-hydroxybenzoic acid (HBA). Inhibition of these carboxylesterases could lead to accumulation of methylparaben (especially if the patient receives large amounts of intravenous solutions) and thus toxicity.

MATERIALS AND METHODS

Substrates and Reagents. The following chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO): chloramphenicol, methylparaben, hydroxybenzoic acid, procaine, p-aminobenzoic acid, procaineamide, prednisolone, prednisolone succinate, paraoxon. Chloramphenicol succinate (The Upjohn Co., Kalamazoo, MI), meperidine (Sterling-Winthrop Research Institute, Rensselaer, NY) and lidocaine hydrochloride (Astra Pharmaceutical Products, Inc., Worcester, MA) were gifts.

Apparatus. All incubations were performed in Dubnoff shaking incubators. Centrifugation of samples after protein denaturation with ethanol was performed with a Fisher Model 59 microcentrifuge (Fisher Scientific, Pittsburgh, PA). The following high-performance liquid chromatography (HPLC) equipment was employed: Waters Model M-6000 isocratic pump (Waters Associates, Milford, MA), Varian Model 2010 isocratic pump (Varian Associates, Sugar Land, TX), Varian Model 2050 variable wavelength ultraviolet spectrophotometer (Varian Associates, Sugar Land, TX), Beckman Model 153 fixed wavelength ultraviolet spectrophotometer (Beckman Corp., Berkeley, CA), Rheodyne Model 7125 injector (Rheodyne, Inc., Cotati, CA). Columns for the HPLC were reversed-phase C-18 prepacked columns obtained from the following companies: Varian Associates, Beckman Corp., Alltech Associates (Deerfield, IL) and Phenomenex hplc Technology (Palos Verdes Estates, CA).

Animals. Adult male guinea pigs were purchased from Charles River Laboratories, Wilmington, MA). All animals were maintained in quarantine for at least 5 days prior to use and were allowed food and water ad libitum Animals were killed by cervical dislocation at the time of use. For those studies in which paraoxon was administered to guinea pigs prior to sacrifice, the paraoxon was prepared for injection by diluting the drug with 95% ethanol and then with distilled water to yield a final ethanol concentration of 47.5%. Paraoxon was administered to different groups of guinea pigs in the following doses: 1) 15 ug/kg, 1 hr prior to sacrifice; 2) 15 ug/kg, 12 hr prior to sacrifice; 3) 150 ug/kg, 1 hr prior to sacrifice; and 4) 150 ug/kg, 12 hr prior to sacrifice. All injections were subcutaneous in the nape of the neck. Although the animals give the higher dose appeared sedated, no overt signs of toxicity were observed.

Tissue Preparation. Tissues (liver, lung and kidney) were removed as rapidly as possible and placed in a pH 7.4 Tris-KCl buffer (0.05 M Tris; 0.15 M KCl) solution at 4°C. Tissues were weighed and minced with scissors. Homogenates were prepared by adding the minced tissue to four parts (weight-volume) Tris-KCl buffer and using a Polytron Homogenizer (Brinkmann Instruments, Inc., Westbury, NY). Homogenates were centrifuged at 9,000 X G for 20 min. The supernatant was transferred and centrifuged at 100,000 X g for 60 min. Supernatants were discarded and the microsomal pellet was resuspended in Tris-KCl buffer and again

centrifuged at 100,000 X g for 60 min. The washed microsomal fraction was transferred to a Potter-Elvehjem tissue grinder and resuspended with the aid of a Teflon pestle. The resuspended microsomal fraction was divided into small fractions and stored at -80°C until used.

Incubation Parameters. Typical conditions for the incubation procedures was as follows: 5 mg of microsomal preparation were added to a 10 ml Teflon beaker containing Tris-KCl buffer (total volume, 4.9 ml). The beaker was placed in an incubator (37°C) and a Teflon ball (1.25 cm diameter) was added to enhance mixing. The mixture was shaken (100 excursions/minute) for 5 min to permit equilibration. At time zero, the substrate (dissolved in 0.10 ml of Tris-KCl) was added to initiate the reaction. At designated times (2.5, 5, 10, 20 and 40 min), 0.10 ml of the incubation mixture was withdrawn from the beaker and processed as described below.

For studies involving the addition of paraoxon to the incubation mixture, the paraoxon was prepared as follows: 20 ul of paraoxon (1.2736 g/ml) was added to 1.0 ml of 95% ethanol; this solution was added to 185 ml of distilled water. After thorough mixing, this aqueous solution was diluted with Tris-KCl to obtain the final solution used for incubations. The concentration of paraoxon in the incubation mixture was usually 0.1 - 10 uM. The final volume of ethanol added to the incubation mixtures was never more than 0.5 ul (maximum concentration in incubation beaker of 0.01% ethanol).

Sample Preparation. At the designated times, an aliquot (0.10 ml) of the incubation mixture was removed and added to a 1.5 ml polycarbonate centrifuge tube (Fisher) containing 0.30 ml of 95% ethanol. The tube was vortexed for 30 sec and then centrifuged for 1 min at 5,000 X g. An aliquot (0.20 ml) of the aqueous-alcohol supernatant was transferred to a screw-capped vial (4 ml capacity) and evaporated to dryness under nitrogen. The residue was resuspended in 0.50 ml of the mobile phase used for the HPLC analysis of that particular substrate (see Appendix 1).

High-Performance Liquid Chromatography. All assays were performed on reversed-phase C-18 prepacked columns with 5 um packing material. The mobile phases for all assays consisted of acetonitrile and phosphate buffer (for specific conditions, see Appendix 1). Quantitation of substrates and products was performed by comparing the peak heights of the extracted samples with peak heights of standards for each compound. Wherever possible, both the substrate and the product were quantitated. This permitted us to estimate the reaction rate from the amount of product formed and from the disappearance of substrate. This method also allowed us to estimate the overall recovery by comparing the amount of added substrate and the combined yield of substrate and product.

RESULTS

HPLC Analysis. Although procedures for the analysis of some of the substrates and their products have been published, all of the procedures used in this investigation were developed in this laboratory. This has allowed us to use procedures which are as uniform as possible so that changeover of the HPLC system from one type of drug to another can be accomplished with a minimum of disruption in the system.

Precision, Recovery and Specificity. In order to establish the validity of the analytical procedures, several studies were performed. The procedures (including incubation and extraction) were the same as those used for the subsequent studies of ester hydrolysis by microsomal preparations. 1) Tissue blank incubations contained buffer and microsomes but no substrate. The results of these studies indicated that no interfering substances were detected by the HPLC analysis of any of the substrates: 2) Substrate blank incubations contained buffer and substrate but no microsomes. These results were used for estimation of recovery and precision of the various assays. Recoveries ranged from 90-110%, and the coefficient of variation of day-to-day results was 4.5% (results not shown).

Linearity of Reaction Rates. Considerable time was devoted to the determination of appropriate parameters for obtaining a linear reaction rate over a particular time interval (usually 20-40 min). These results are shown in Figures 1-4 for the four substrates which undergo ester hydrolysis under the conditions described.

The hydrolysis of chloramphenicol succinate was found to be linear over 20 min at the following substrate concentrations (Figure 1): 0.25 mM, 0.5 mM and 1.0 mM. Similar results were obtained with prednisolone succinate (Figure 2) over the same concentration range. Since procaine is hydrolyzed more rapidly than these two substrates, a wider range of substrate concentrations was employed. These results indicate that the hydrolysis of procaine is not linear at 0.1 mM but is linear at 0.4, 0.8 and 1.6 mM mM concentrations (Figure 3). The hydrolysis of methylparaben is quite distinct from that of the other substrates. Although the reaction is linear over 20 minutes at several substrate concentrations, the rate of the reaction is the same at all substrate concentrations (Figure 4). These results suggest that the hydrolysis of methylparaben follows zero- order kinetics rather than first-order kinetics, as is usually the case.

The microsomal preparation from guinea pig liver failed to hydrolyze three of the substrates tested : lidocaine, procaineamide and meperidine.

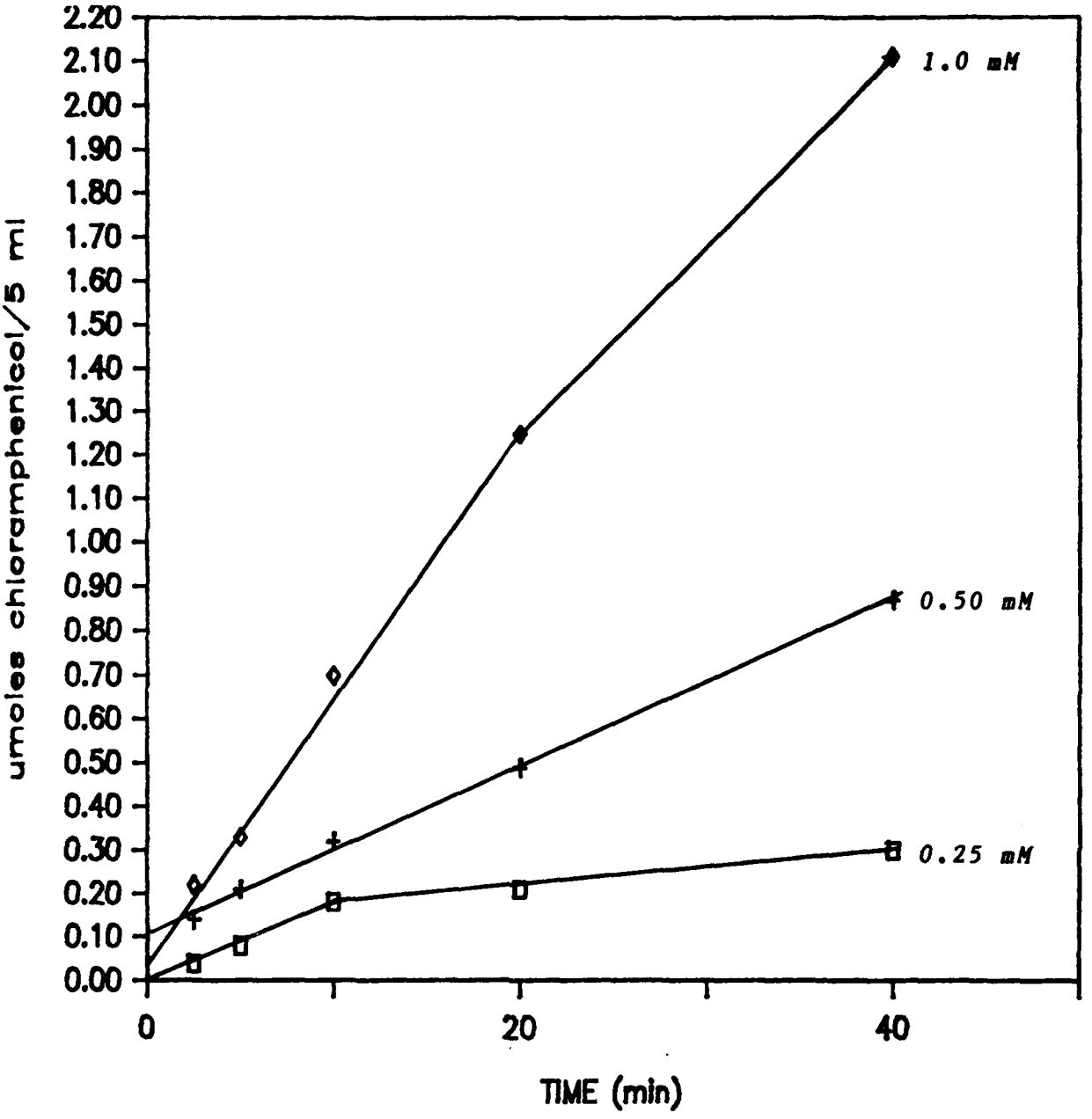

Figure 1. Hydrolysis of Chloramphenicol Succinate. Data are expressed as umoles of chloramphenicol formed from the incubation of chloramphenicol succinate with 5 mg of microsomal protein obtained from the liver of guinea pigs. Volume of the incubation was 5 ml.

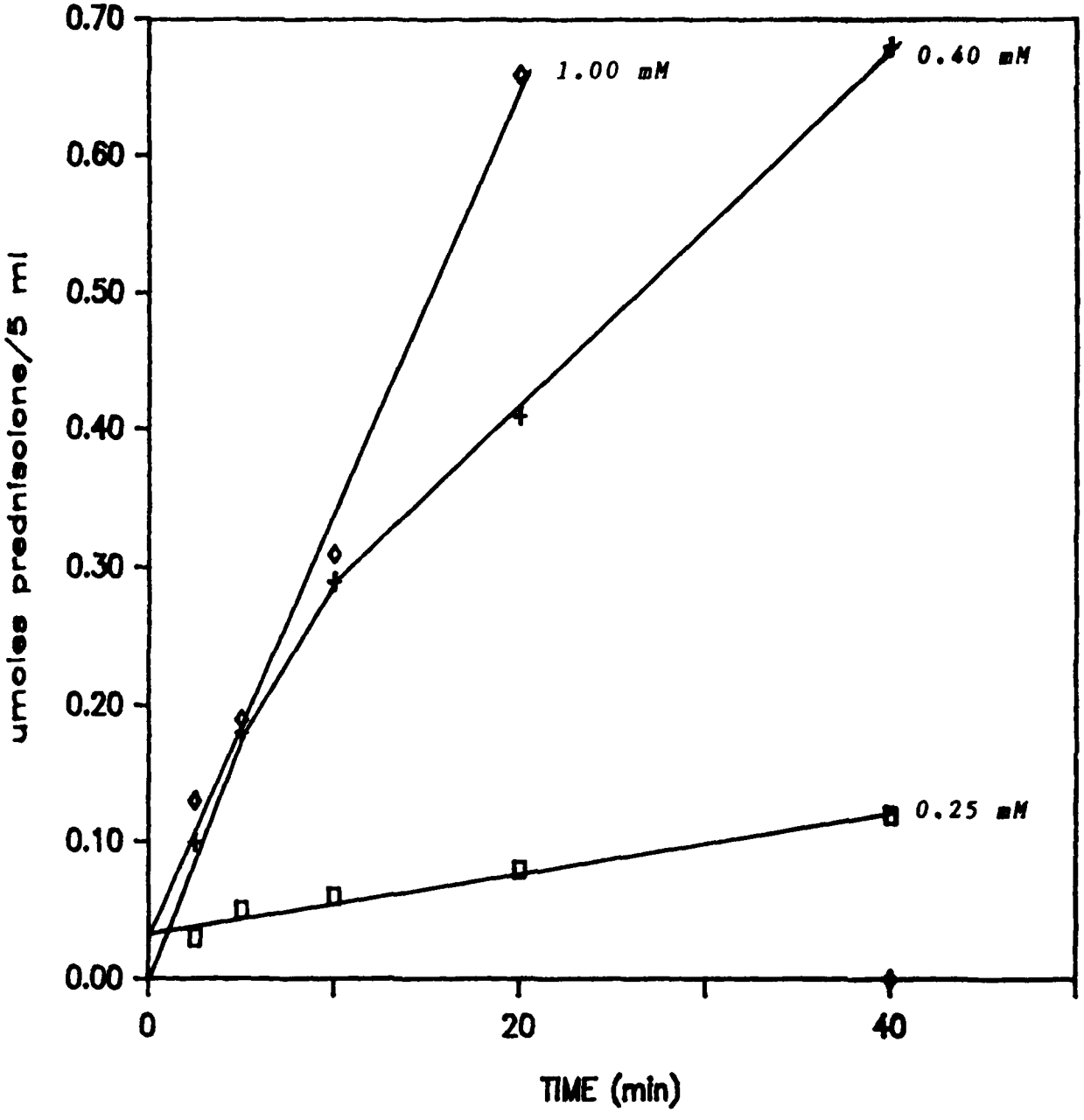

Figure 2. Hydrolysis of Prednisolone Succinate. Data are expressed as umoles of prednisolone formed from the incubation of prednisolone succinate with 5 mg of microsomal protein obtained from the liver of guinea pigs. Volume of the incubation was 5 ml.

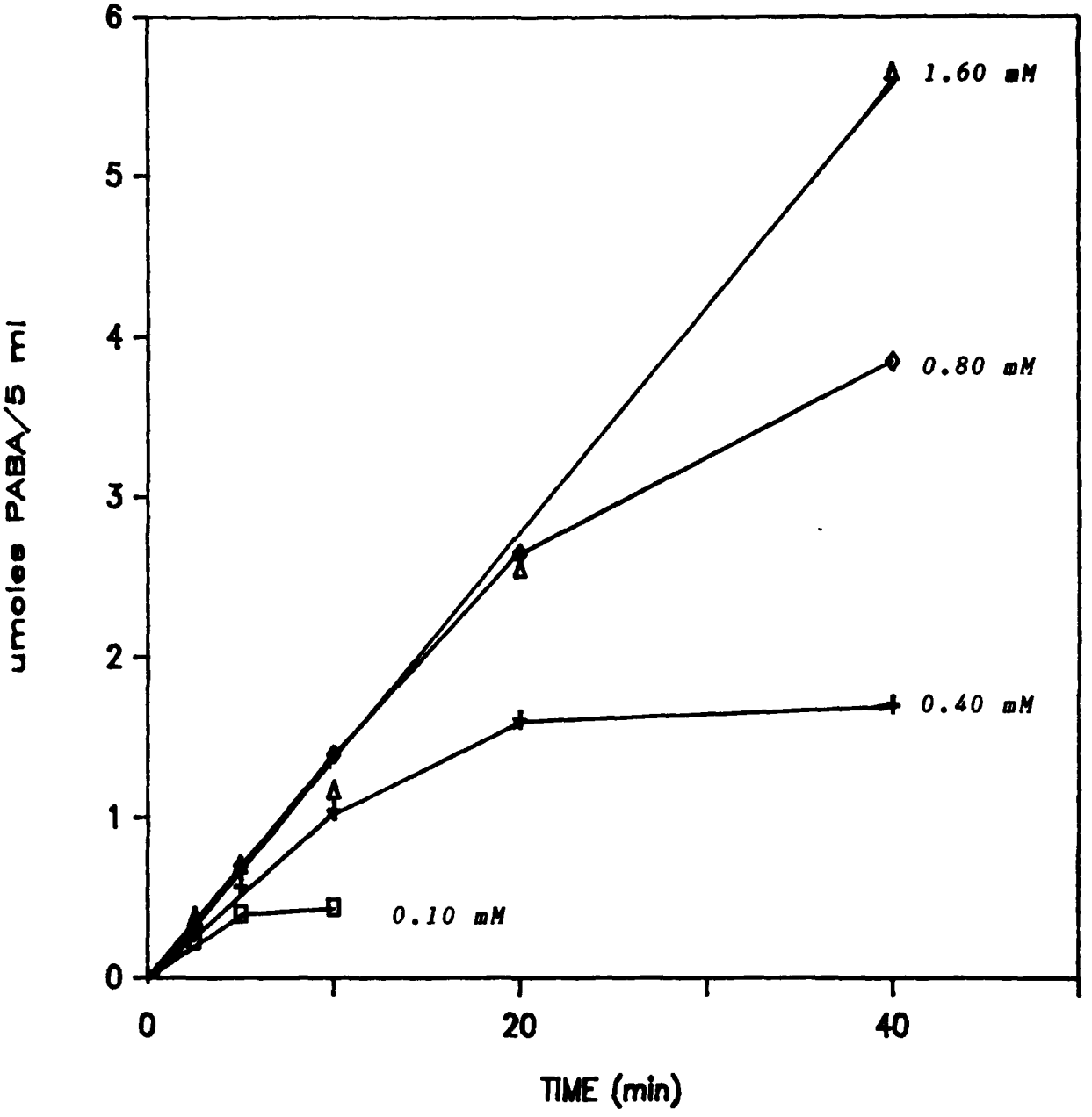

Figure 3. Hydrolysis of Procaine. Data are expressed as umoles of p-aminobenzoic acid (PABA) formed from the incubation of procaine with 5 mg of microsomal protein obtained from the liver of guinea pigs. Volume of the incubation was 5 ml.

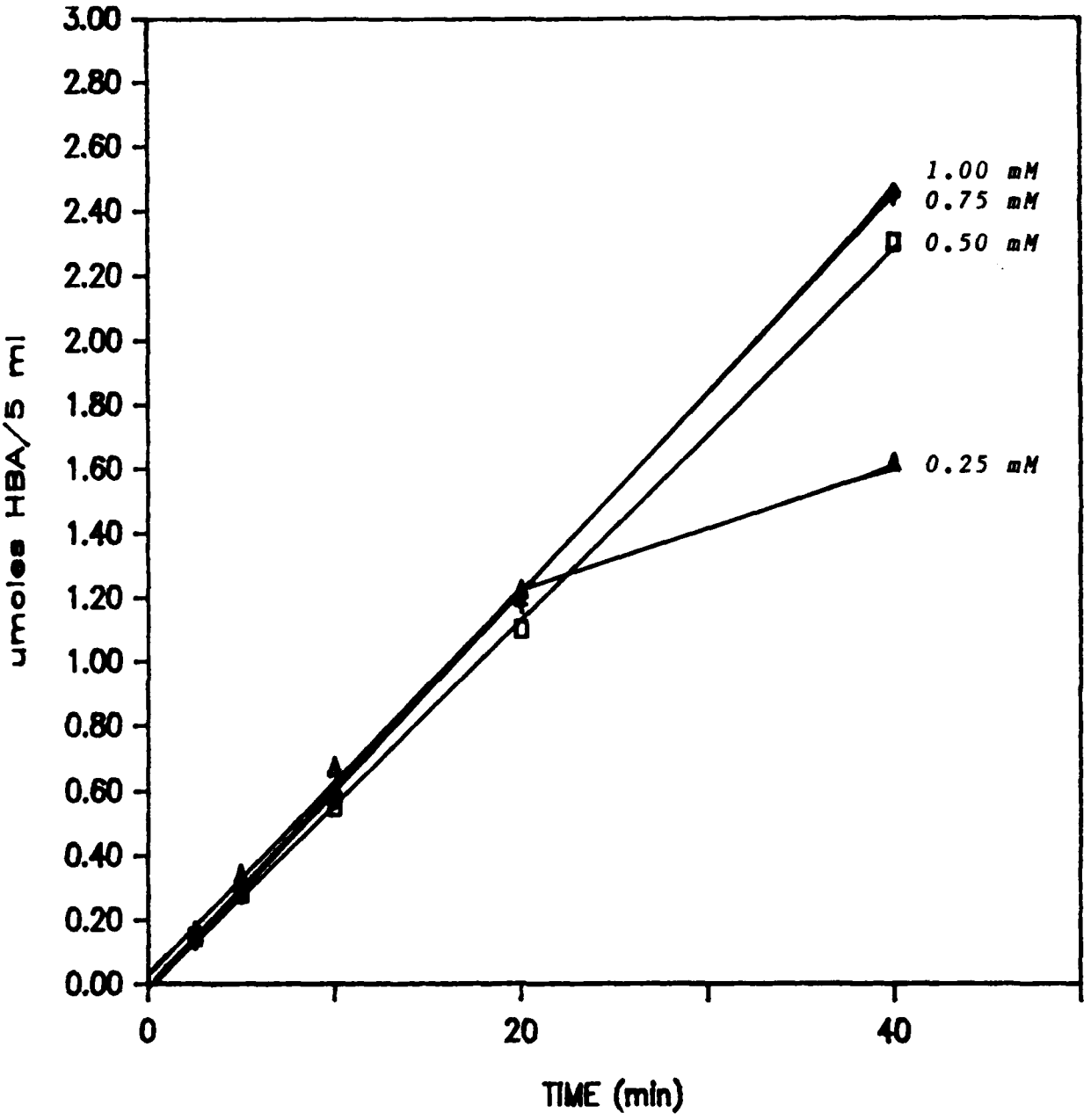

Figure 4. Hydrolysis of Methylparaben. Data are expressed as umoles of hydroxybenzoic acid (HBA) formed from the incubation of methylparaben with 5 mg of microsomal protein obtained from the liver of guinea pigs. Volume of the incubation was 5 ml.

Inhibition of Ester Hydrolysis by Paraoxon In Vitro . The addition of paraoxon to the incubation mixtures resulted in inhibition of ester hydrolysis for all substrates.

The inhibitory effect of paraoxon on the hydrolysis of chloramphenicol succinate and prednisolone succinate were similar (Tables 1 and 2). As indicated in the Lineweaver-Burk double reciprocal plots (Figures 5 and 6), increasing concentrations of paraoxon produced parallel lines with similar slopes.

Table 1

Hydrolysis of Chloramphenicol Succinate

Paraoxon (uM)	Km (mM)	Vmax (um/min/5 mg protein)
0	0.802	0.153
0.10	1.059	0.165
0.75	0.441	0.053
1.00	0.187	0.022

Table 2

Hydrolysis of Prednisolone Succinate

Paraoxon (uM)	Km (mM)	Vmax (um/min/5 mg protein)
0	0.627	0.143
0.25	0.569	0.107
0.50	1.420	0.220
0.625	0.625	0.050
0.75	0.270	0.024
1.00	0.218	0.014

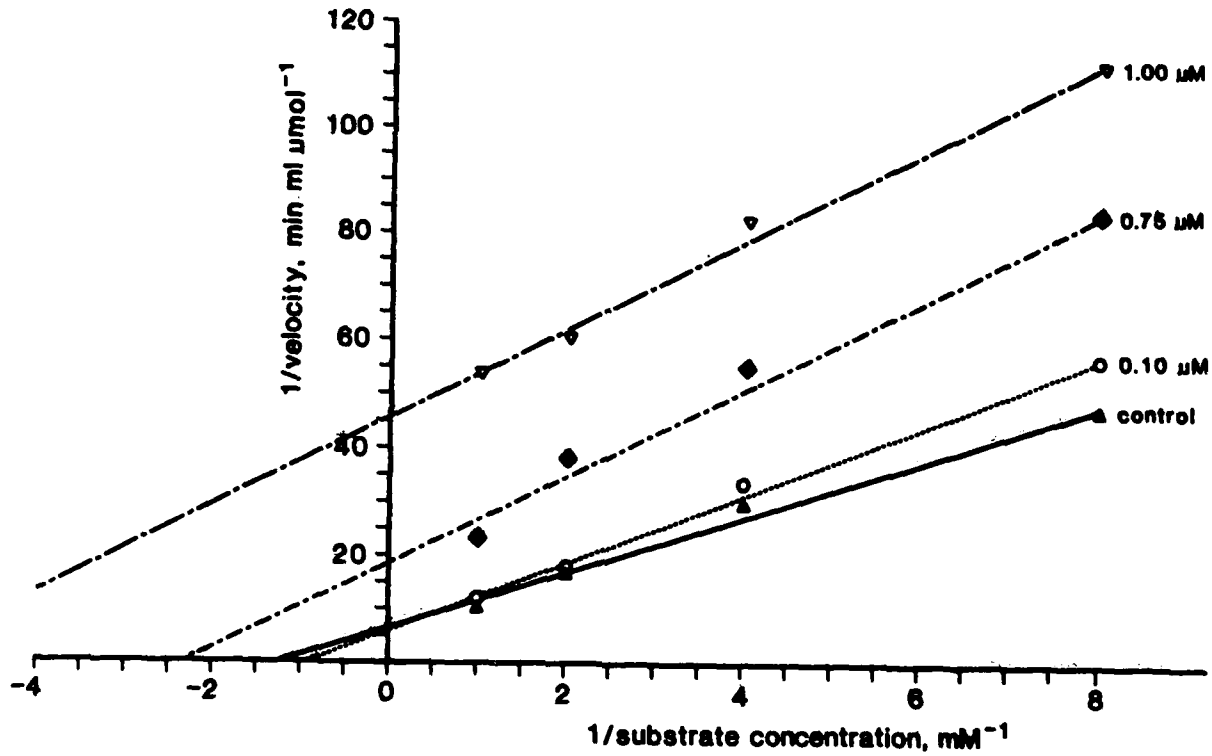

Figure 5. Inhibition of Hydrolysis of Chloramphenicol Succinate by Paraoxon. Various concentrations of substrate (0.10, 0.75,and 1.00 mM) were incubated for 10 min in the presence of the indicated concentrations of paraoxon.

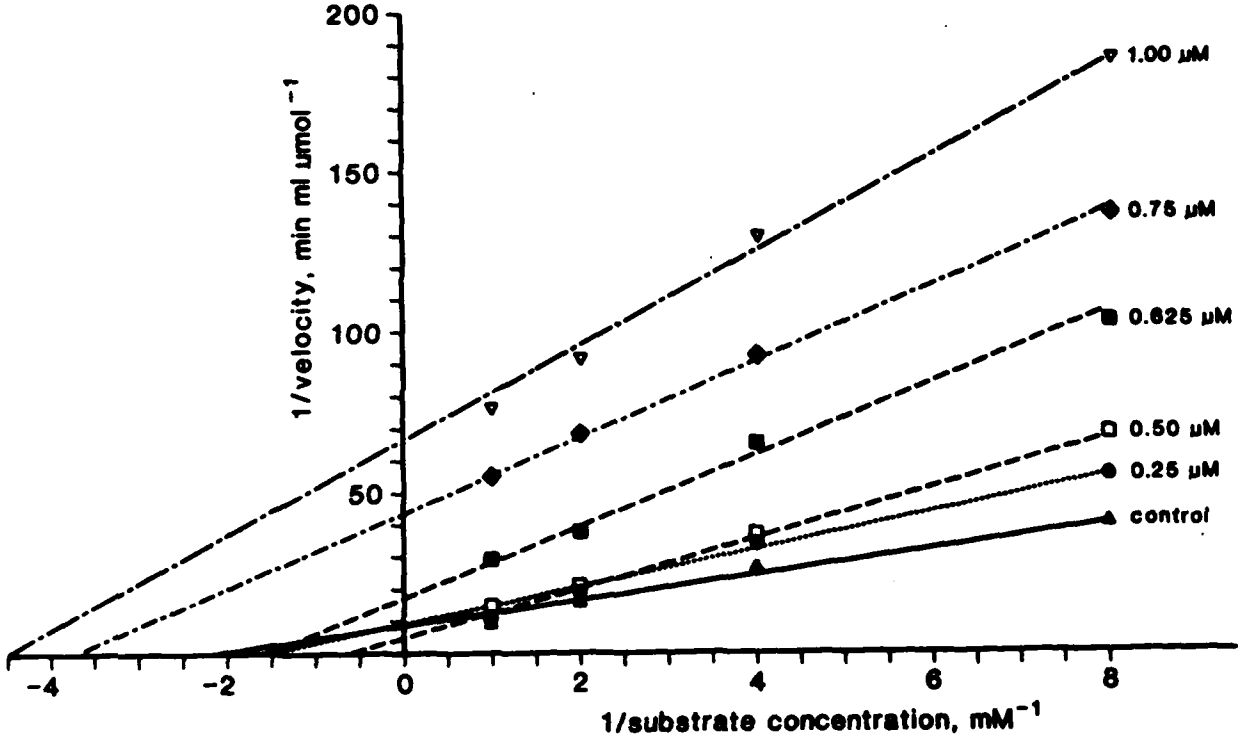

Figure 6. Inhibition of Hydrolysis of Prednisolone Succinate by Paraoxon. Various concentrations of substrate (0.25, 0.50, 0.625, 0.75 and 1.00 mM) were incubated for 10 min in the presence of the indicated concentrations of paraoxon.

The rate of hydrolysis of procaine was the most rapid of the four substrates (Table 3). The addition of various concentrations of paraoxon produced a plot with markedly different slopes but approximately the same intersection of the Y-axis (Figure 7).

Since the hydrolysis followed zero-order kinetics under the present conditions (Figure 4), kinetic analysis is not meaningful. As shown in Table 4 and Figure 8, the effect of adding paraoxon is to produce lines with negative slopes.

Table 3

Hydrolysis of Procaine

Paraoxon (uM)	Km (mM)	Vmax (um/min/5 mg protein)
0	0.192	0.259
0.50	0.195	0.137
0.75	0.355	0.126
1.00	0.645	0.093
1.25	2.511	0.094

Table 4

Hydrolysis of Methylparaben

Paraoxon (uM)	Km (mM)	Vmax (um/min/5 mg protein)
0	-0.074	0.042
0.50	-0.090	0.035
0.75	-0.101	0.025
1.00	-0.070	0.020
1.25	2.175	0.022

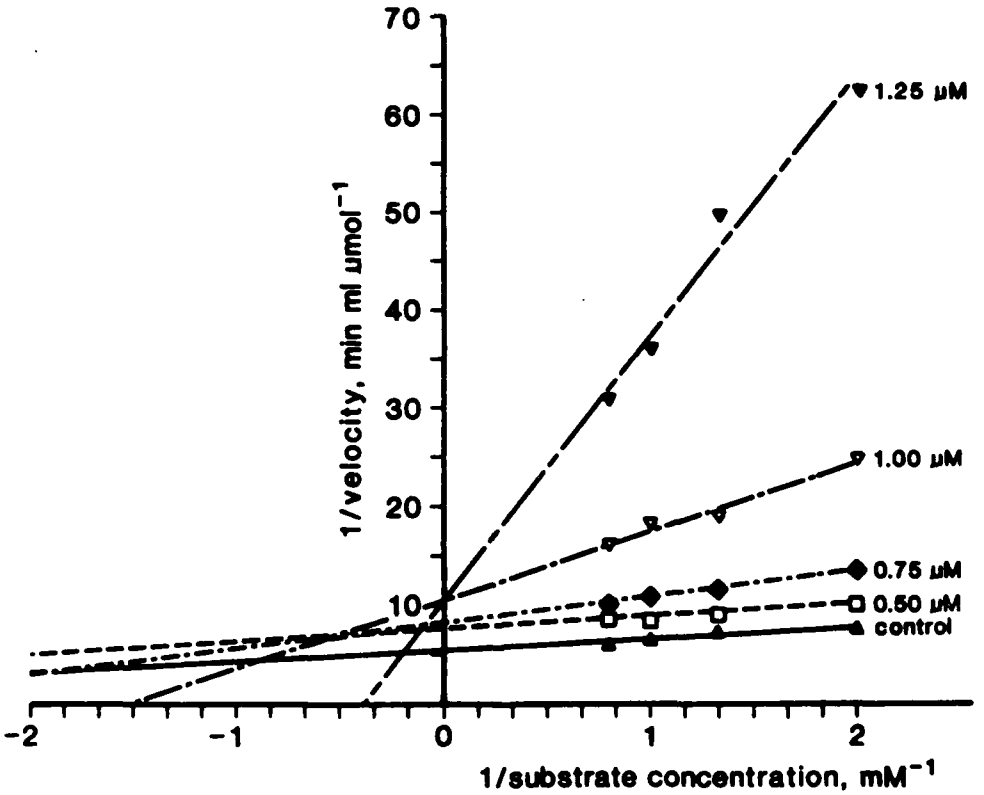

Figure 7. Inhibition of Hydrolysis of Procaine by Paraoxon. Various concentrations of substrate (0.5, 0.75, 1.00 and 1.25 mM) were incubated for 10 min in the presence of the indicated concentrations of paraoxon.

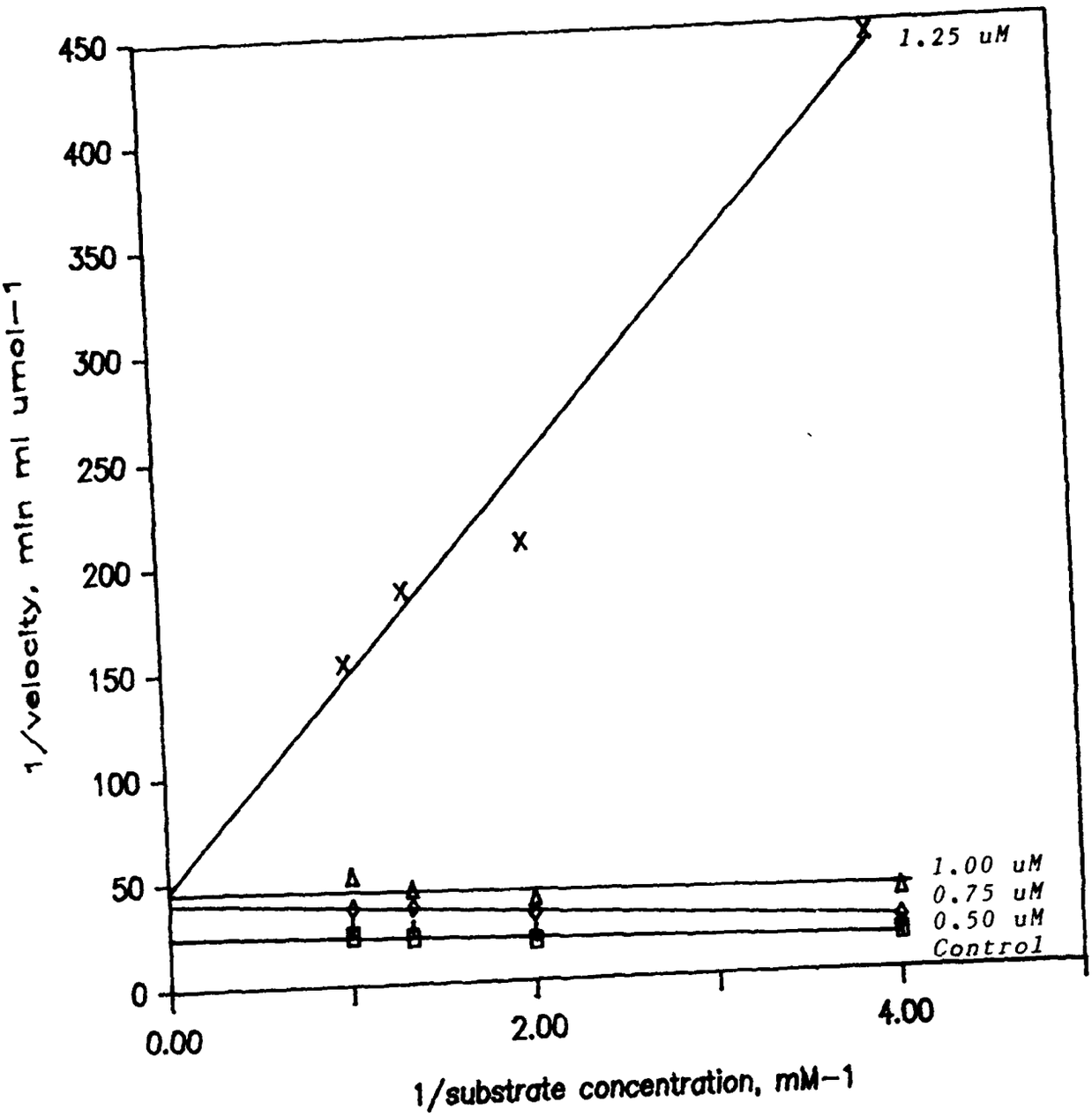

Figure 8. Inhibition of Methylparaben by Paraoxon. Various concentrations of substrate (0.50, 0.75, 1.00 and 1.25 mM) were incubated for 20 min in the presence of the indicated concentrations of paraoxon.

Inhibition of Ester Hydrolysis by Paraoxon Pretreatment. The last phase of the scheduled work for the first year of the contract consists of the administration of paraoxon to guinea pigs in order to assess its effects on ester hydrolysis. After administration of paraoxon, tissues (liver, lung and kidney) were removed and microsomes were isolated by the usual procedures. The contract specifies two doses (approximately 0.1 and 1 LD50) and two time intervals between paraoxon administration and sacrifice (1 hr and 12 hr). Since it was not possible to perform all incubations for a given animal on a single day, the potential change in the inhibitory effects of paraoxon during storage of microsomes, even at -80°C was a source of concern. In order to determine whether there were changes in esterase activity with storage, reaction rates were determined immediately after preparation of microsomes and then at monthly intervals. No changes have been observed in hydrolysis rates with storage. When soman is studies in the second phase of this project, a standardized carboxylesterase assay will be used to determine the effects of storage on esterase activity of microsomal preparations from control and soman-treated guinea pigs. This assay will be used to quantitate ester hydrolysis at various time intervals after the preparation of microsomal fractions. In this assay the hydrolysis of p-nitrophenylacetate is quantitated by spectrophotometric analysis of the product (p-nitrophenol).

The microsomal preparations from control and paraoxon-treated guinea pigs were used to study the rates of hydrolysis of four drug esters: chloramphenicol succinate, prednisolone succinate, procaine and methylparaben. For each substrate, studies were performed to determine the linearity of the reaction at different substrate concentrations. The results of these studies are presented as plots in Appendix 2. The results of the enzyme kinetic studies are summarized in Tables 5-7 and Figures 9-11.

These data suggest that the rate of hydrolysis of chloramphenicol succinate by the liver-microsomal fraction is inhibited only at the higher paraoxon concentration (150 ug/kg) at both 1 hr and 12 hr after administration of the inhibitor. However, by 12 hr the extent of inhibition was reduced by half. This inhibition is paralleled by decreases in the affinity of the enzyme for the substrate. The hydrolysis rates for the kidney were only about 20 percent of those of the liver and very little inhibition was observed after the administration of paraoxon.

Table 5

Enzyme Kinetics of Chloramphenicol Succinate Hydrolysis

	Liver		Kidney	
Treatment	Km (mM)	Vmax (um/min/5 mg protein)	Km (mM)	Vmax (um/min/5 mg protein)
Control	1.151	0.145	0.352	0.023
Paraoxon 1 hr (15 ug/kg)	1.206	0.141	0.270	0.034
Paraoxon 12 hr (15 ug/kg)	0.902	0.169	0.238	0.026
Paraoxon 1 hr (150 ug/kg)	0.725	0.106	*	*
Paraoxon 12 hr (150 ug/kg)	0.859	0.125	0.464	0.030

* Indicates that no incubations were performed because of insufficient amount of tissue.

Table 6

Enzymes Kinetics of Prednisolone Succinate Hydrolysis

Treatment	Km (mM)	Vmax (um/min/5 mg protein)
Control	0.443	0.083
Paraoxon 1 hr (15 ug/kg)	0.275	0.087
Paraoxon 12 hr (15 ug/kg)	0.579	0.067
Paraoxon 1 hr (150 ug/kg)	0.598	0.129
Paraoxon 12 hr (150 ug/kg)	0.347	0.078

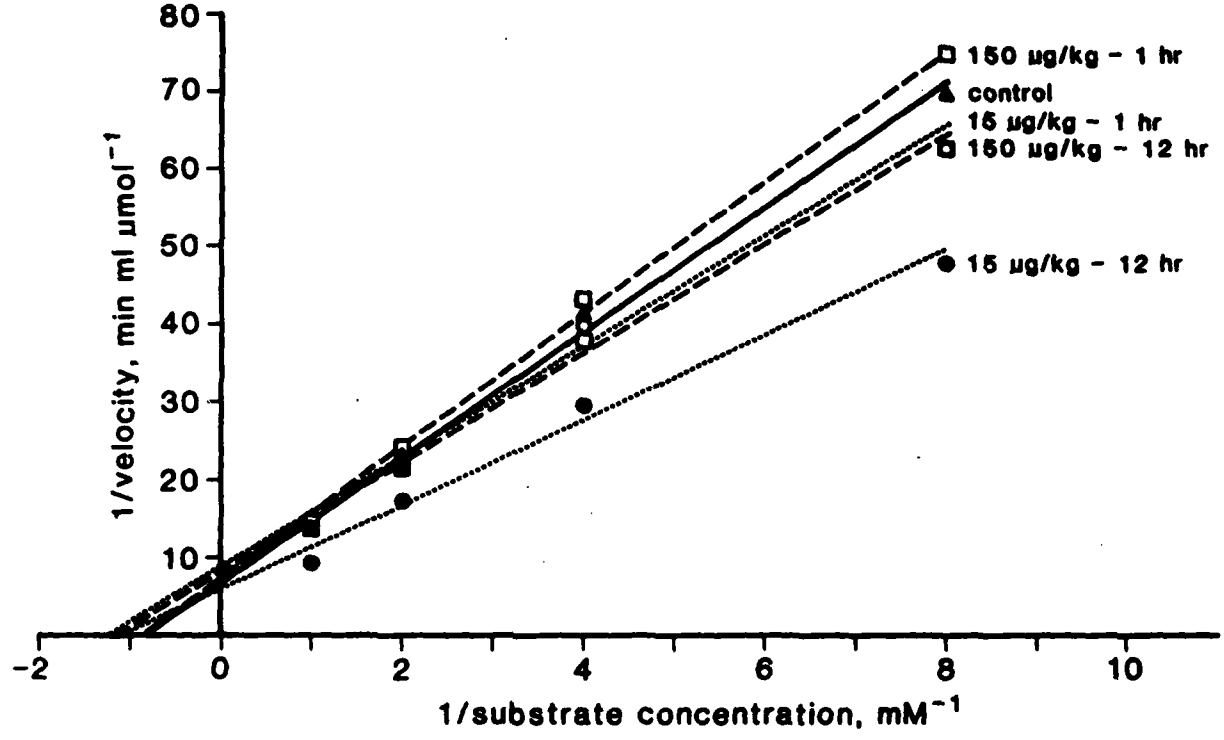

Figure 9. Effect of Paraoxon Pretreatment on the Hydrolysis of Chloramphenicol Succinate by Liver Microsomes.

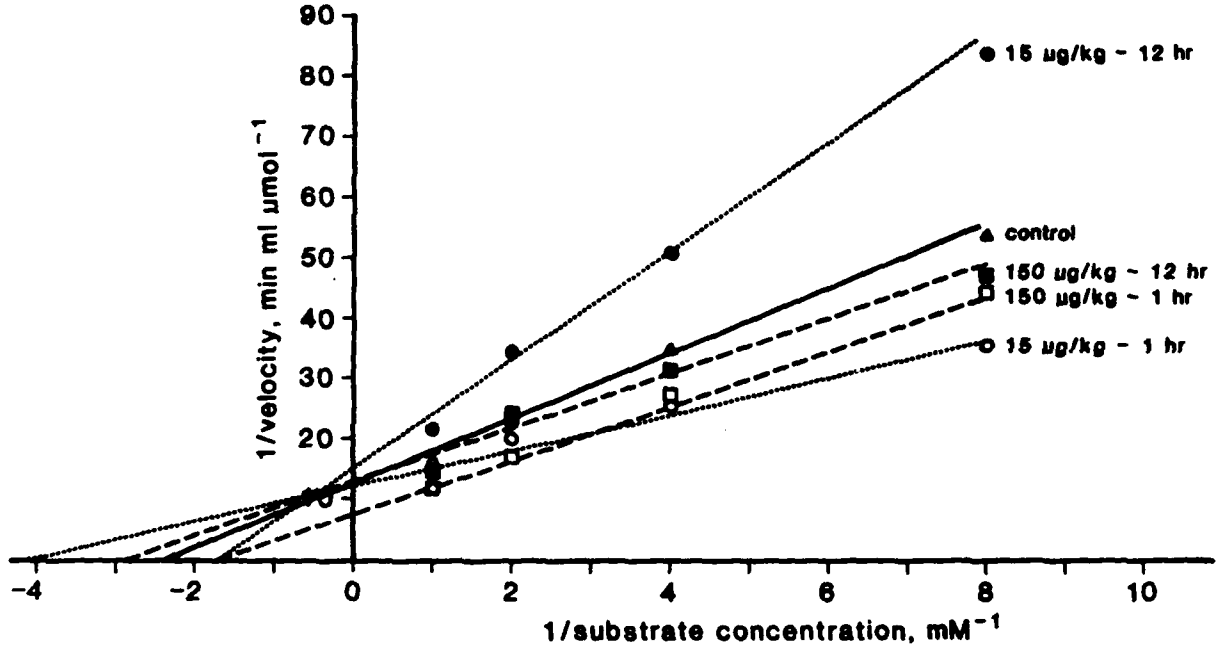

Figure 10. Effect of Paraoxon Pretreatment on the Hydrolysis of Prednisolone Succinate by Liver Microsomes.

The administration of paraoxon had very little effect on the hydrolysis rate of prednisolone succinate by the liver (Table 6). Some inhibition did occur 12 hr after administration of the lower dose and there appeared to be an increase in the hydrolysis rate 1 hr after the higher dose.

The data in Table 7 suggest that the inhibition of the rate of procaine hydrolysis in the liver is similar to that of chloramphenicol succinate except that the inhibitory effects of the higher dose of paraoxon are sustained for a longer time. Again, no inhibition was seen in the kidney.

Table 7

Enzyme Kinetics of Procaine Hydrolysis

	Liver		Kidney	
Treatment	Km (mM)	Vmax (um/min/5 mg protein)	Km (mM)	Vmax (um/min/5 mg protein)
Control	0.175	0.170	0.121	0.033
Paraoxon 1 hr (15 ug/kg)	0.084	0.141	0.197	0.016
Paraoxon 12 hr (15 ug/kg)	0.274	0.194	*	*
Paraoxon 1 hr (150 ug/kg)	0.031	0.132	0.104	0.039
Paraoxon 12 hr (150 ug/kg)	0.111	0.121	0.194	0.027

*Indicates no incubations were performed because of insufficient amount of tissue.

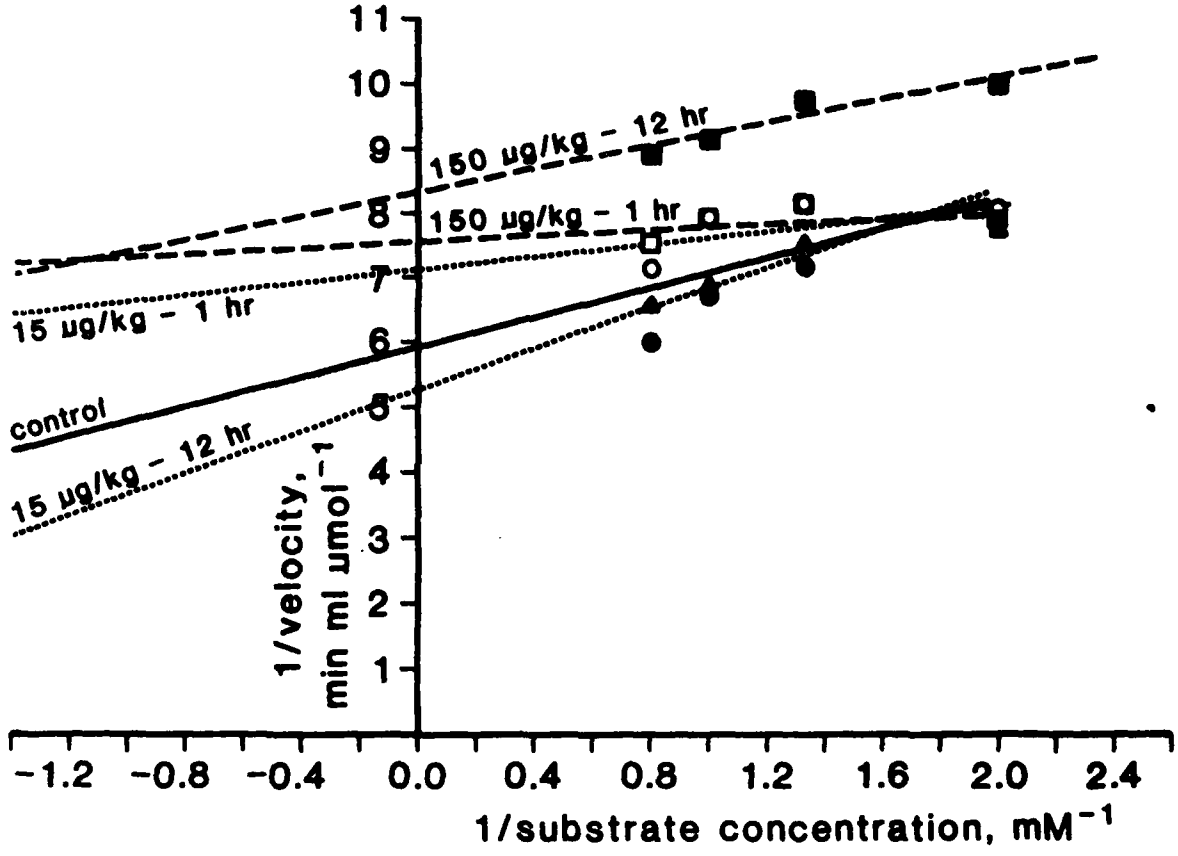

Figure 11. Effect of Paraoxon Pretreatment on the Hydrolysis of Procaine by Liver Microsomes.

DISCUSSION

In the reported investigations, we have addressed the problem of an appropriate animal model for characterization of esterase enzymes responsible for the metabolism of carboxylester drugs. Sporadic reports of the hydrolysis of these drugs by esterases have appeared in the literature over the years. However, no systematic approach has been reported to determine the role of these carboxylesterases in the metabolism of drugs. The question of whether the same esterases are responsible for the hydrolysis of amide esters and carboxylesters has not been adequately addressed. Even less information is available concerning inhibition of these esterases (e.g., by organophosphate compounds) and the consequences of such inhibition to the overall disposition of esters which are widely used as antibiotics, antiarrhythmic agents, antiinflammatory agents, analgesics, anesthetics and preservatives in intravenous solutions.

Chloramphenicol succinate, the form of the antibiotic for intravenous administration, is hydrolyzed in the liver, lung and kidney to release the active component, chloramphenicol. Our data suggest that this hydrolysis is markedly inhibited by the presence of paraoxon, both in vitro and in vivo. The Lineweaver-Burk (Figure 5) suggests that this inhibition is of the type sometimes referred to as uncompetitive inhibition, anticompetitive inhibition and coupling inhibition. In this situation, the inhibitor can couple only with the enzyme-substrate complex and not with the free enzyme. The effect of this inhibition is to produce a family of straight lines which parallel that for the uninhibited reaction, thus producing proportional changes in the Vmax and Km.

Prednisolone succinate, another prodrug used for intravenous injection, is likewise inhibited by organophosphate compounds. The Lineweaver-Burk plot (Figure 6) is similar to that of chloramphenicol succinate except at the lowest inhibitor concentration (0.10 uM). Thus, inhibition of the hydrolysis of prednisolone succinate appears to follow coupled inhibition pattern.

The results of the inhibition of procaine hydrolysis by paraoxon (Figure 7) suggest purely competitive inhibition. The effect of this type of inhibition is to decrease the affinity of the enzyme for the substrate.

The hydrolysis of methylparaben by esterases of the liver of guinea pigs constitutes a distinct type of reaction not observed with the other substrates discussed above. Studies to establish the linearity of the reaction indicated that the amount of product (p-hydroxybenzoic acid) formed is independent of the substrate concentration. This property is typical of zero-order reactions, in which a constant quantity of substrate is converted per unit of time. The more common reactions are first-order:

i.e., a constant percentage of the remaining substrate is converted per unit of time. When a Lineweaver-Burk plot (Figure 8) is constructed for methylparaben in the presence of the inhibitor, the Km has a negative value, since the slopes of the lines are the reverse of those usually observed.

The rate of hydrolysis of all four substrates by lung and kidney microsomes was considerably less than that of the liver, usually 5 to 20 percent of that observed with liver tissue (see Appendix 2 for specific data). In general, reaction rates were less in lung than in kidney. The substrate most readily hydrolyzed by the lung was methylparaben, which followed zero-order kinetics, as observed previously with liver tissue. Neither prednisolone succinate nor procaine was hydrolyzed to any significant extent by lung tissue. Kidney tissue hydrolyzed all substrates except prednisolone succinate at approximately the same rate. These results suggest that the major site of hydrolysis for all of these substrates is the liver.

The results discussed above indicate that paraoxon inhibits microsomal carboxylesterase activity in a dose-dependent manner. To investigate this effect in vivo, paraoxon was administered to guinea pigs at a low dose (10 percent of an LD50) or at a high dose (90 percent of an LD50) either 1 hr or 1 hr prior to sacrifice of the animals. No consistent pattern of enzyme inhibition was observed and in some pretreated groups, the rate of hydrolysis increased by as much as 55 percent that of the control group. These results may indicate that competing processes of enzyme inhibition and enzyme enhancement are produced by the administration of paraoxon to guinea pigs. A more consistent and pronounced enhancement of carboxylesterase activity has been demonstrated by pretreatment of guinea pigs with soman, another organophosphate compound which is a more potent inhibitor of carboxylesterase in vitro than is paraoxon (data presented in Annual Report of May, 1986).

SUMMARY AND CONCLUSIONS

The results of these investigations demonstrate that carboxylesterase activity toward several esters of drugs is present in liver, lung and kidney. The liver is by far the most important site of hydrolysis of these ester compounds. Some substrates are hydrolyzed by the lung and kidney to a greater extent than other substrates.

No amidase activity was observed toward the two substrates tested (lidocaine and procaineamide) with microsomal preparation from guinea pig liver. Some investigators have suggested that a single enzyme or group of enzymes is responsible for hydrolysis of both carboxylesters and esters with amide linkages (1). The results of the present studies do not support this proposal.

Although five of the substrates tested contain carboxylester linkages (chloramphenicol succinate, prednisolone succinate, procaine, methylparaben and meperidine), there were marked differences among these substrates in the characteristics of their hydrolysis by microsomal preparations of guinea pig liver. Since chloramphenicol succinate and prednisolone succinate contain the same ester linkage, it is predictable that these two substrates would be hydrolyzed similarly, as was demonstrated in this research. This similarity extends to inhibition as well as uninhibited hydrolysis.

Procaine is probably the best-characterized substrate for carboxylesterase enzyme activity. In these studies, procaine was the best substrate for the carboxylesterase enzymes, with reaction rates 2 to 5 times greater than those for the other substrates. The activity of these enzymes toward procaine was markedly inhibited by the presence of paraoxon.

An unexpected finding in these investigations was the inability of microsomal preparations from guinea pig liver to hydrolyze meperidine. This drug has been reported to be a good substrate for carboxylesterases of rat liver (5). No hydrolysis of meperidine was observed during 40-min incubation of several substrate concentrations. In order to determine whether this represents a species difference, microsomal preparations from rat liver and mouse liver were also tested. Both of these preparations hydrolyzed meperidine extensively. These data suggest that the carboxylesterases of the guinea pig liver possess different substrate specificities from those of the rat or mouse.

The administration of paraoxon, even in high doses, failed to produce a consistent or pronounced inhibition of carboxylesterase activity. In some cases, pretreatment with paraoxon actually increased enzyme activity. These results suggest paraoxon pretreatment may cause either inhibition or enhancement of enzyme activity in the guinea pig.

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APPENDIX 1: HPLC PARAMETERS

The analysis the various substrates and products by high-performance liquid chromatography (HPLC) was performed on three different HPLC instruments using columns from several manufacturers. However, certain conditions were common to all assays. All columns were reversed-phase columns and contained C-18 packing material with a 5-uM particle size. Mobiles phases for all assays consisted of acetonitrile in phosphate buffer. The buffer for all assays consisted of 25 mM monobasic potassium phosphate (KH_2PO_4). The final solution was adjusted to pH 4.0 with phosphoric acid.

1) Chloramphenicol.
Column: Beckman Ultrasphere C-18
Mobile Phase: 30% acetonitrile
Flow Rate: 1.5 ml/min
UV Detector: 278 nm or 254 nm
Sensitivity: 0.02 attenuation units full-scale (AUFS)
Injection Volume: 50 ul

2) Prednisolone.
Column: Alltech C-18 or Beckman Ultrasphere C-18
Mobile Phase: 30% acetonitrile
Flow Rate: 1.5 ml/min
UV Detector: 254 nm
Sensitivity: 0.02 AUFS
Injection Volume: 50 ul

3) Procaine.
Column: Alltech Econosphere C-18
Mobile Phase: 10% acetonitrile
Flow Rate: 1.5 ml/min
UV Detector: 254 nm
Sensitivity: 0.02 AUFS
Injection Volume: 50 ul

4) Methylparaben.
Column: Alltech Econosphere C-18
Mobile Phase: 22% acetonitrile
Flow Rate: 1.5 ml/min
UV Detector 254 nm
Sensitivity: 0.02 AUFS
Injection Volume: 50 ul

5) Lidocaine.
Column: Econosphere C-18 or Varian C-18
Mobile Phase: 10% acetonitrile
Flow Rate: 1.5 ml/min
UV Detector: 214 nm
Sensitivity: 0.02 AUFS
Injection Volume: 50 ul

6) Meperidine.

Column: Econosphere C-18
Mobile Phase: 50% acetonitrile in buffer of 25 mM potassium phosphate and 10 mM sodium perchlorate, pH 4.0.
Flow Rate: 1.5 ml/min
UV Detector: 214 nm
Sensitivity: 0.02 AUFS
Injection Volume: 50 ul

7) Procaineamide.

Column: Alltech Econosphere C-18
Mobile Phase: 10% acetonitrile
Flow Rate: 1.5 ml/min
UV Detector: 254 nm
Sensitivity: 0.02 AUFS
Injection Volume: 50 ul

APPENDIX 2: FIGURES FOR ENZYME KINETICS

This section contains figures for studies performed on tissues (liver, lung and kidney) obtained from guinea pigs which received paraoxon (15 ug/kg or 150 ug/kg) either 1 hr or 12 hr before sacrifice. Two types of plots are presented: 1) Lineweaver-Burk plots for lung and kidney - only a limited number of these were performed because of lack of sufficient amount of tissue; 2) Linearity - in general, two or more concentrations of substrate were investigated for linearity of hydrolysis with time.

The conditions of the incubation procedure were similar for all studies: 5 mg microsomal protein in a Teflon beaker containing a total volume of 5 ml.

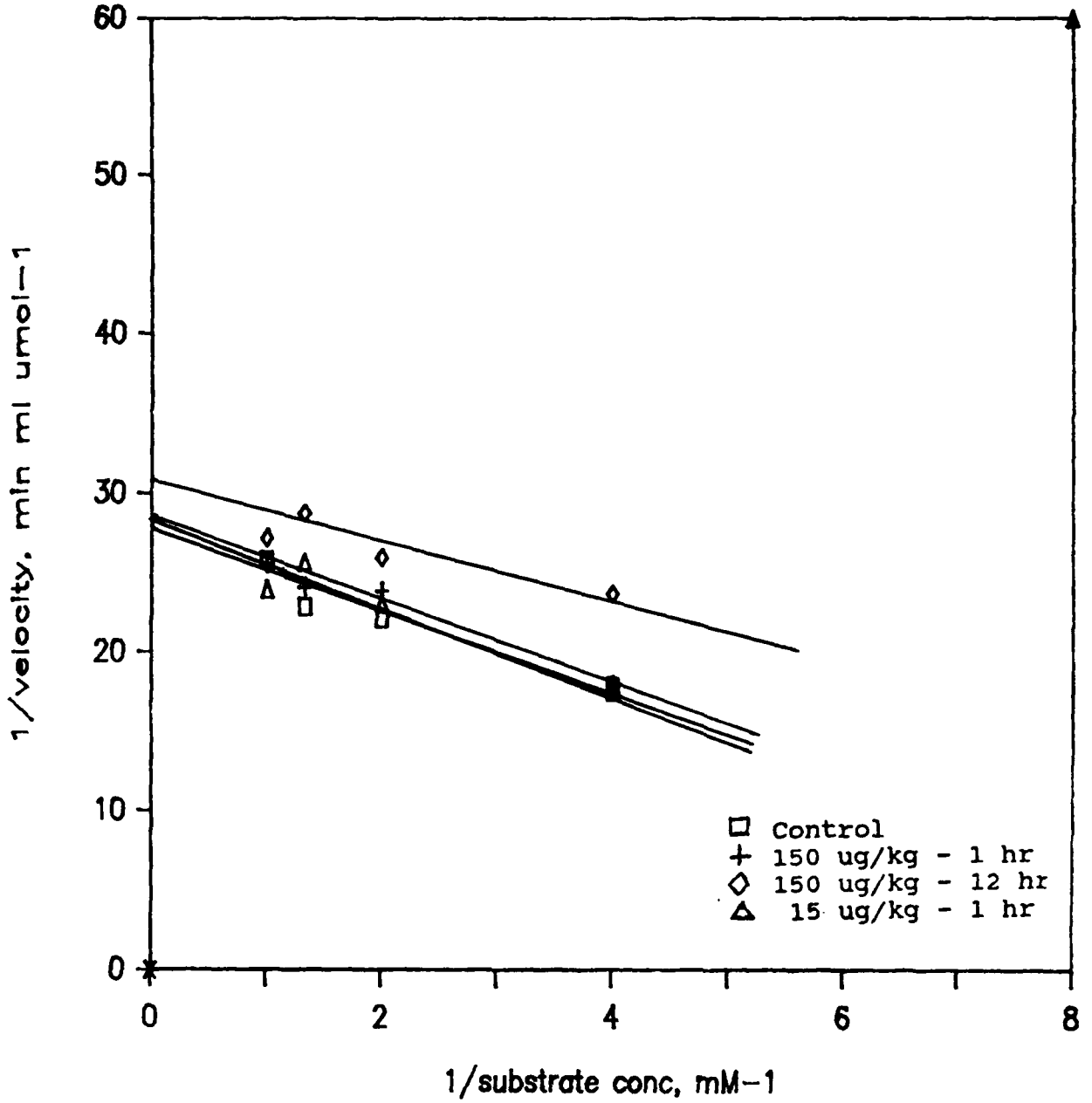

Figure 12. Effect of Paraoxon Pretreatment on the Hydrolysis of Methylparaben by Liver Microsomes.

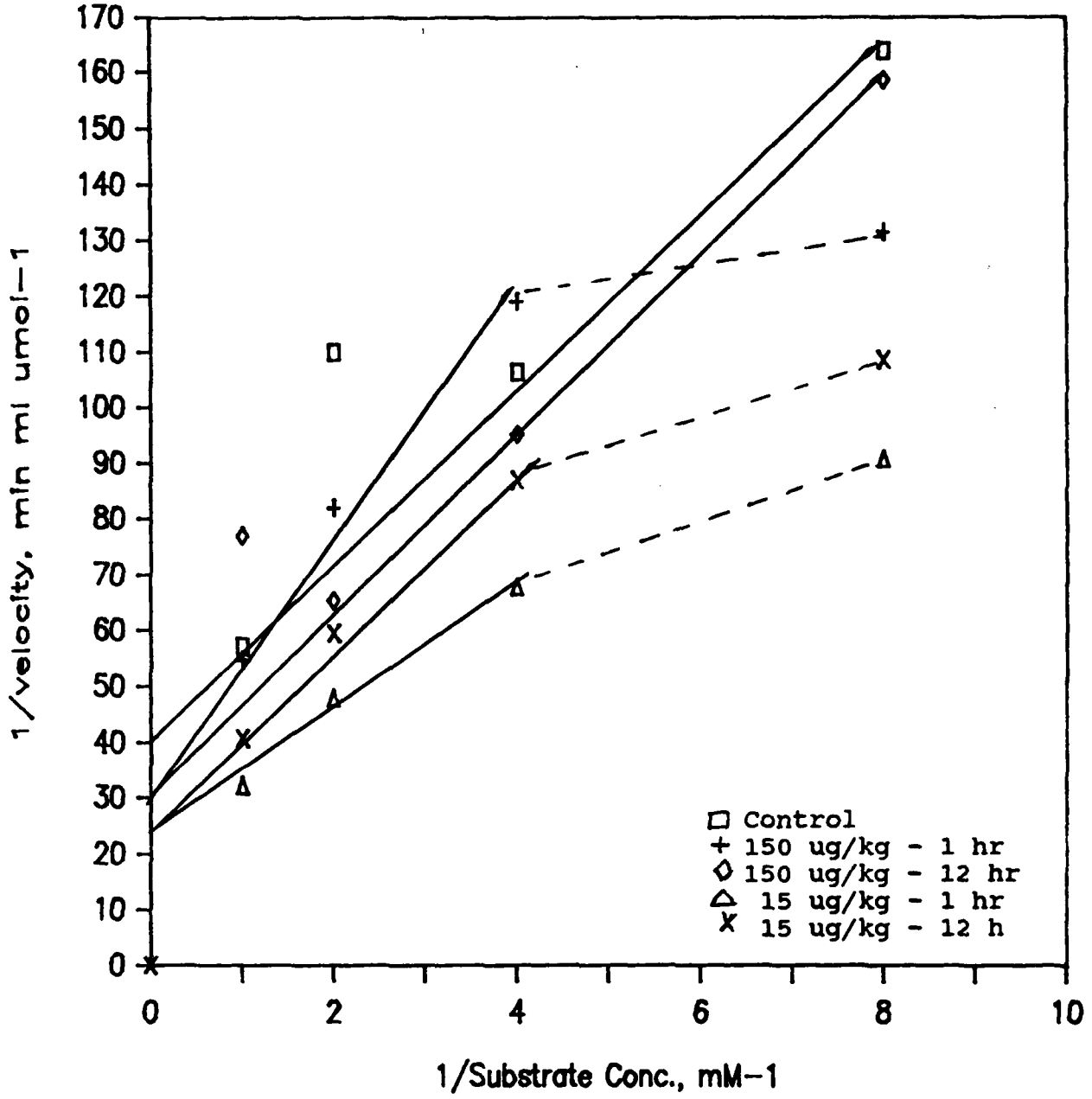

Figure 13. Effect of Paraoxon Pretreatment on the Hydrolysis of Chloramphenicol Succinate by Kidney Microsomes.

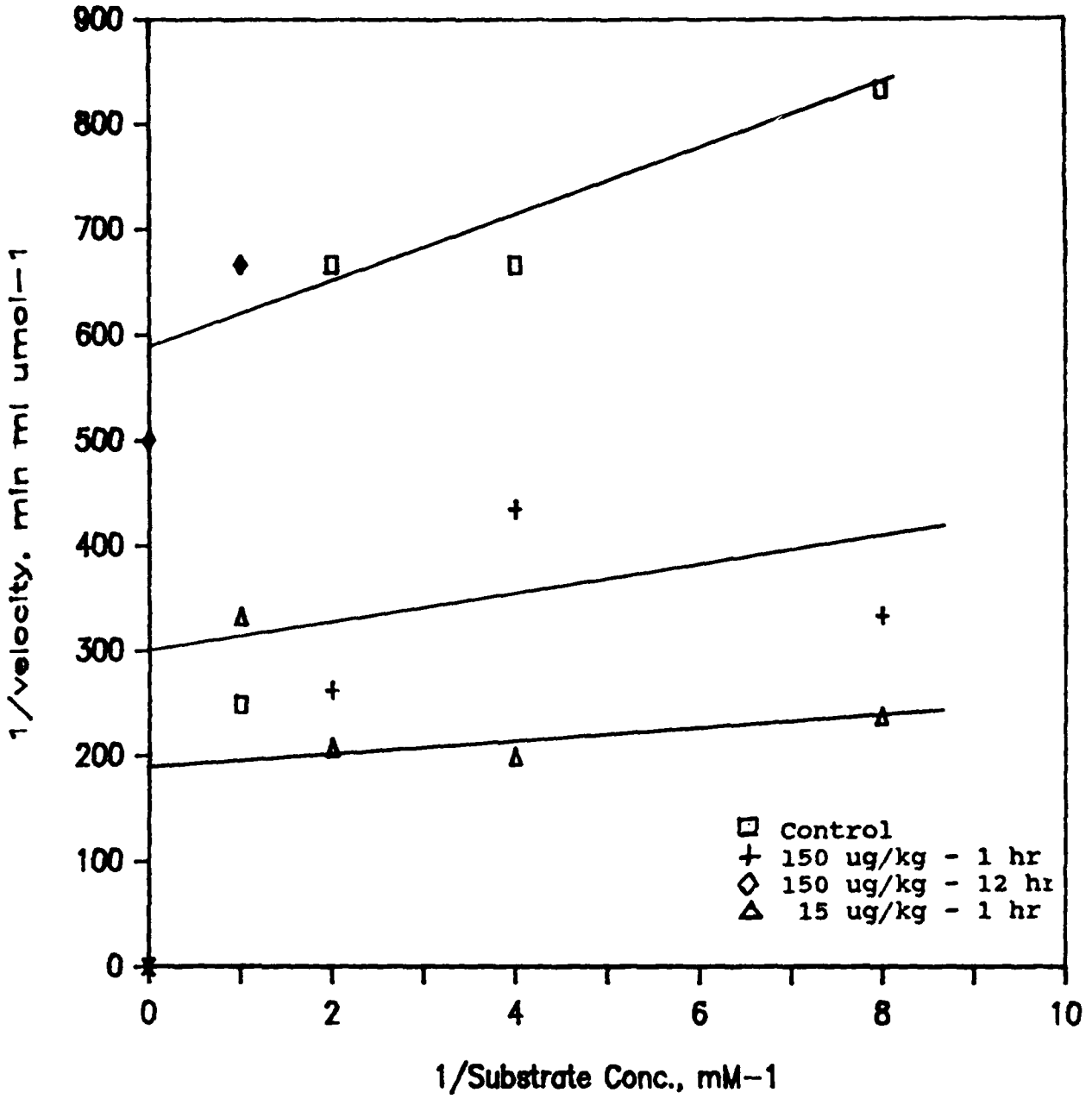

Figure 14. Effect of Paraoxon Pretreatment on the Hydrolysis of Prednisolone Succinate by Kidney Microsomes.

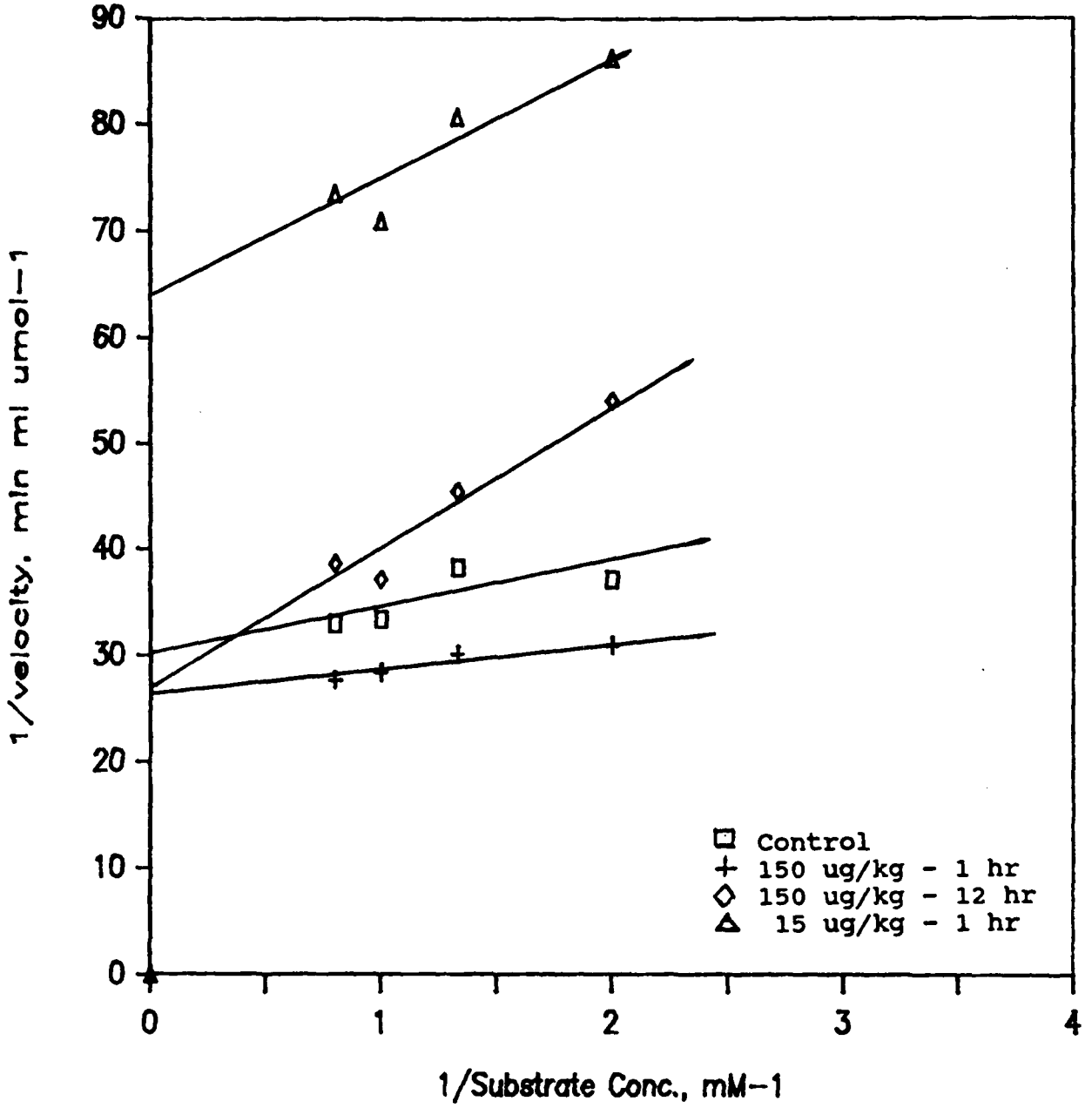

Figure 15. Effect of Paraoxon Pretreatment on the Hydrolysis of Procaine by Kidney Microsomes.

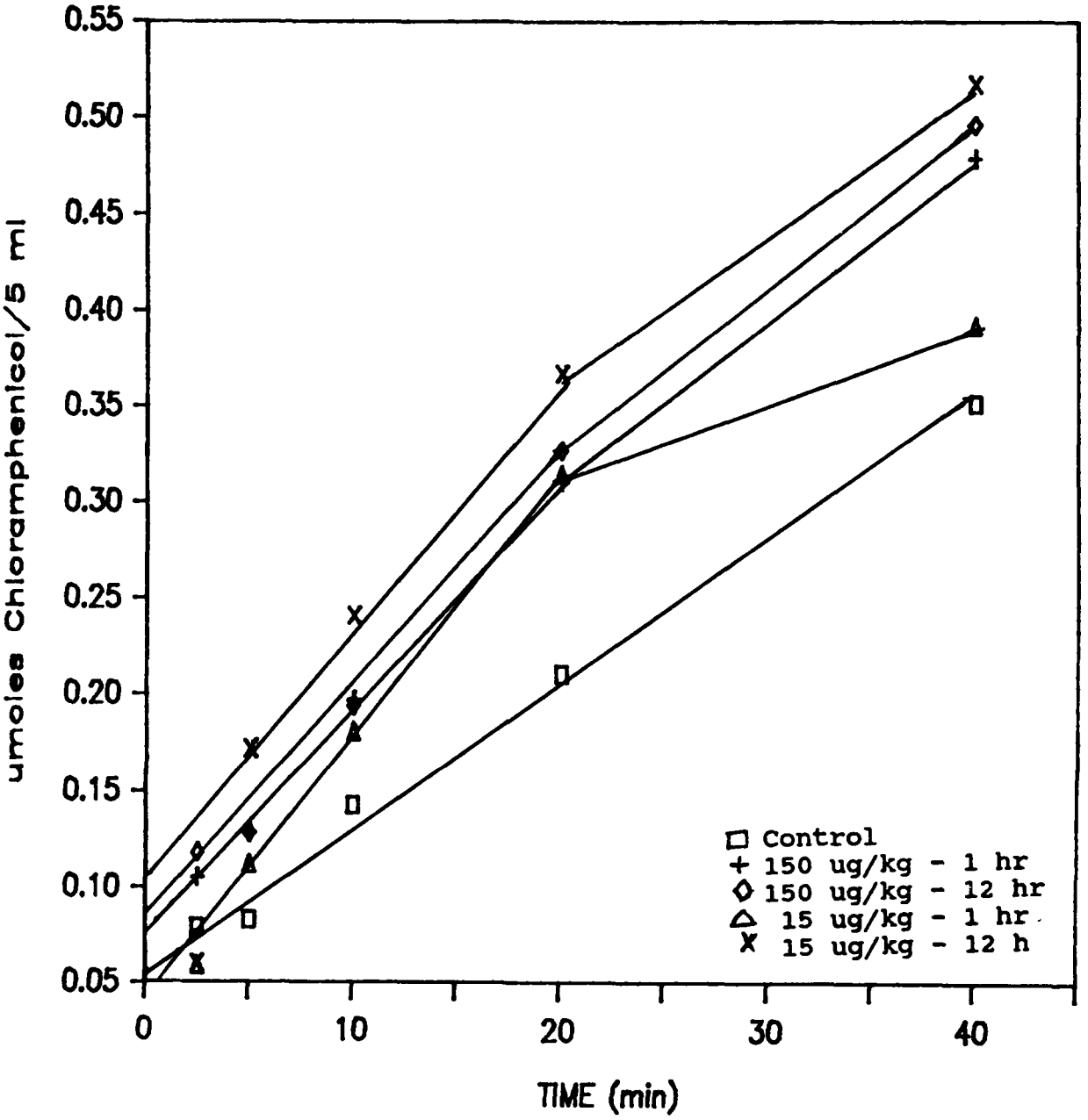

Figure 16. Linearity of the Hydrolysis of Chloramphenicol Succinate by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Chloramphenicol succinate (0.125 mM) was incubated with 5 mg of microsomal protein.

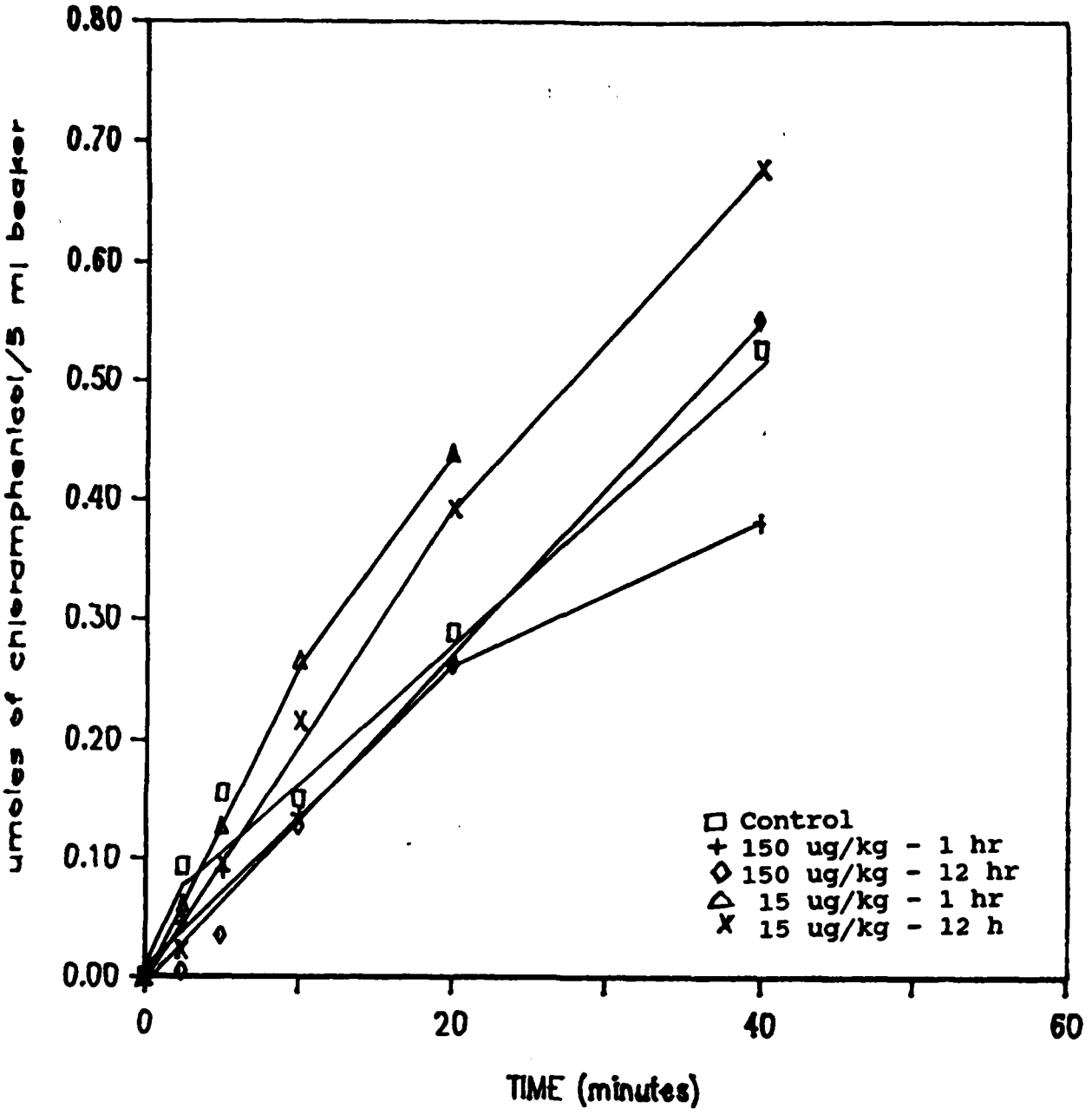

Figure 17. Linearity of the Hydrolysis of Chloramphenicol Succinate by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Chloramphenicol succinate (1.00 mM) was incubated with 5 mg of microsomal protein.

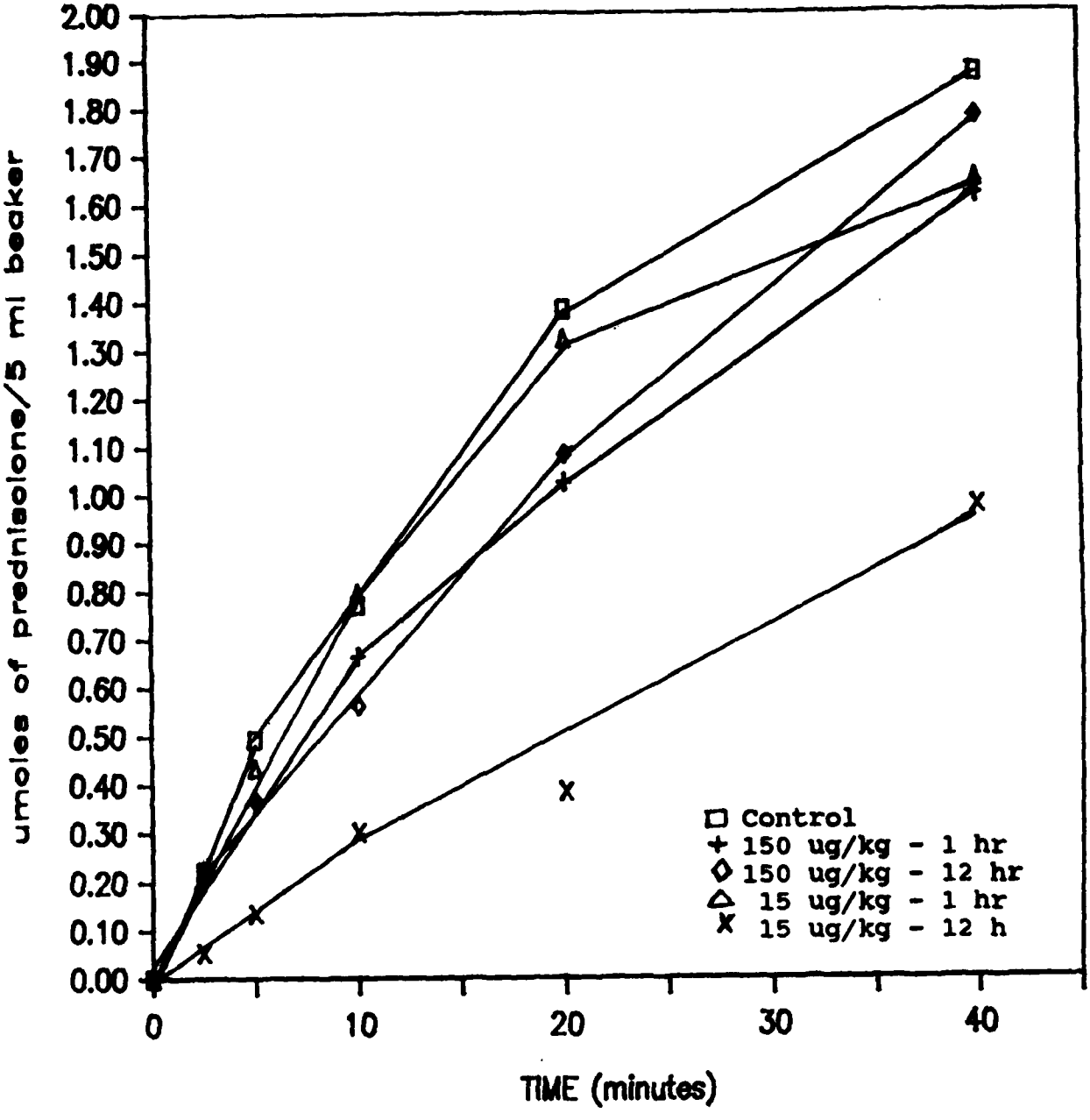

Figure 18. Linearity of the Hydrolysis of Prednisolone Succinate by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Prednisolone succinate (0.125 mM) was incubated with 5 mg of microsomal protein.

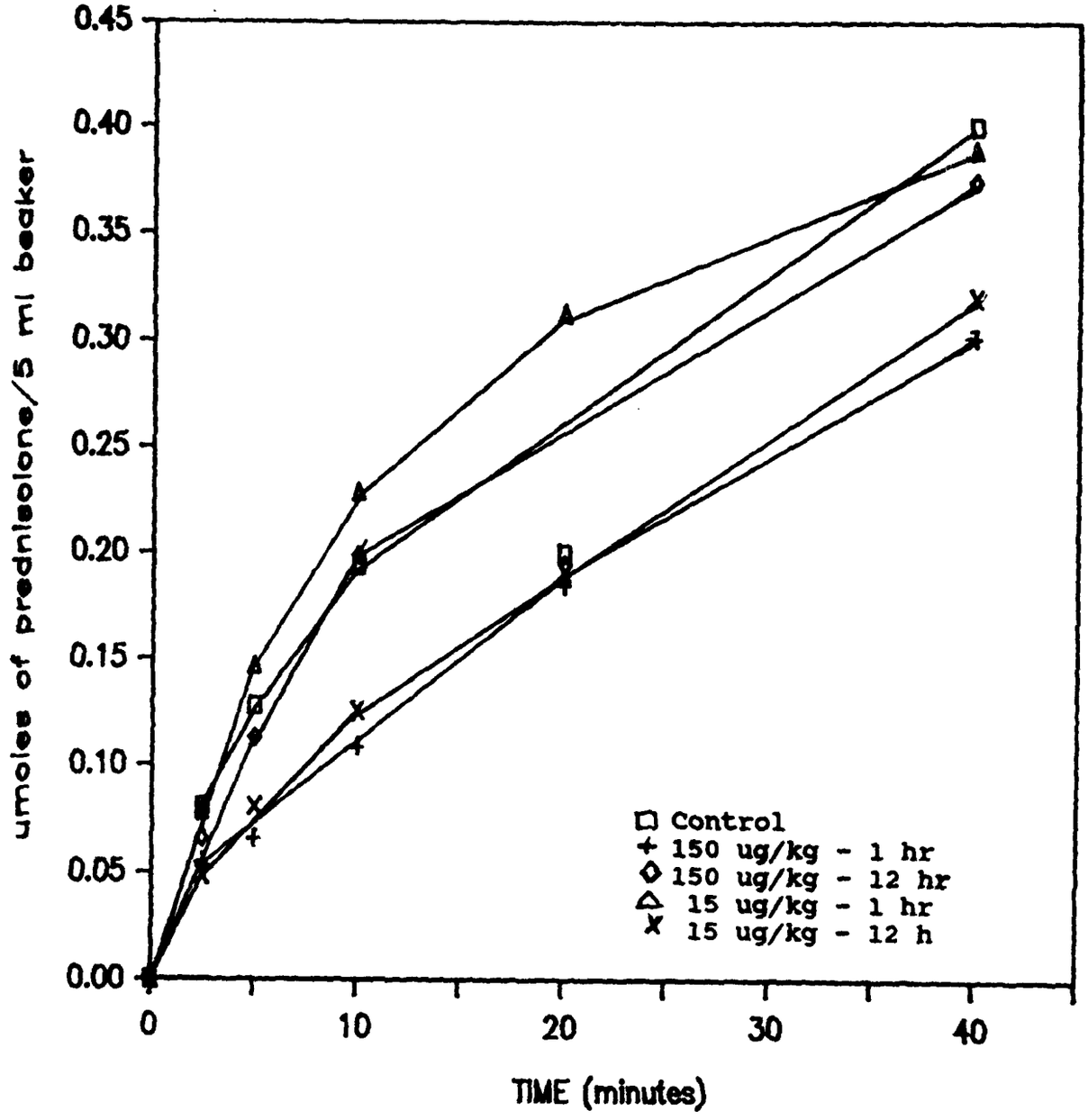

Figure 19. Linearity of the Hydrolysis of Prednisolone Succinate by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Prednisolone succinate (1.00 mM) was incubated with 5 mg of microsomal protein.

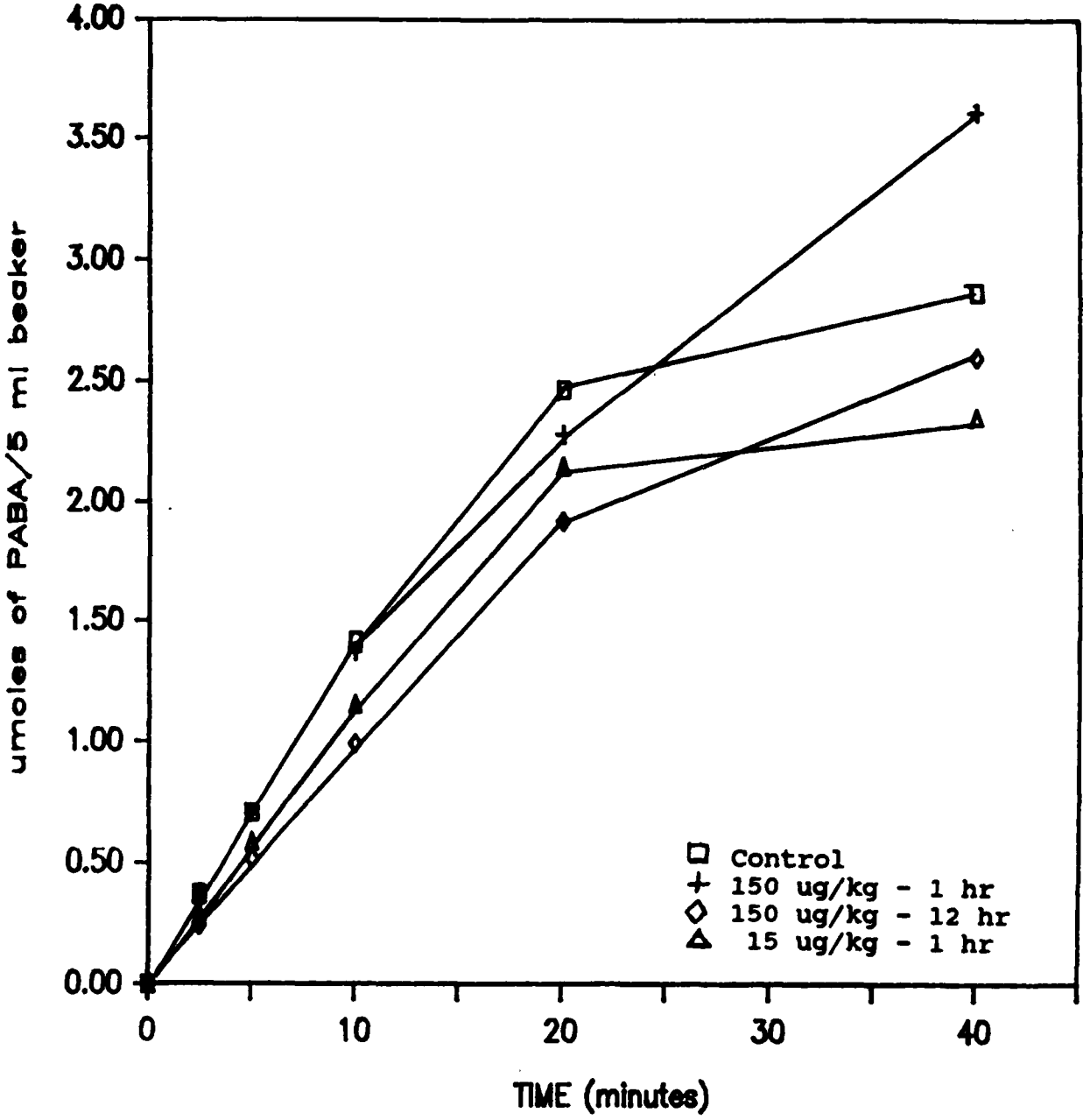

Figure 20. Linearity of the Hydrolysis of Procaine by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Procaine (0.50 mM) was incubated with 5 mg of microsomal protein.

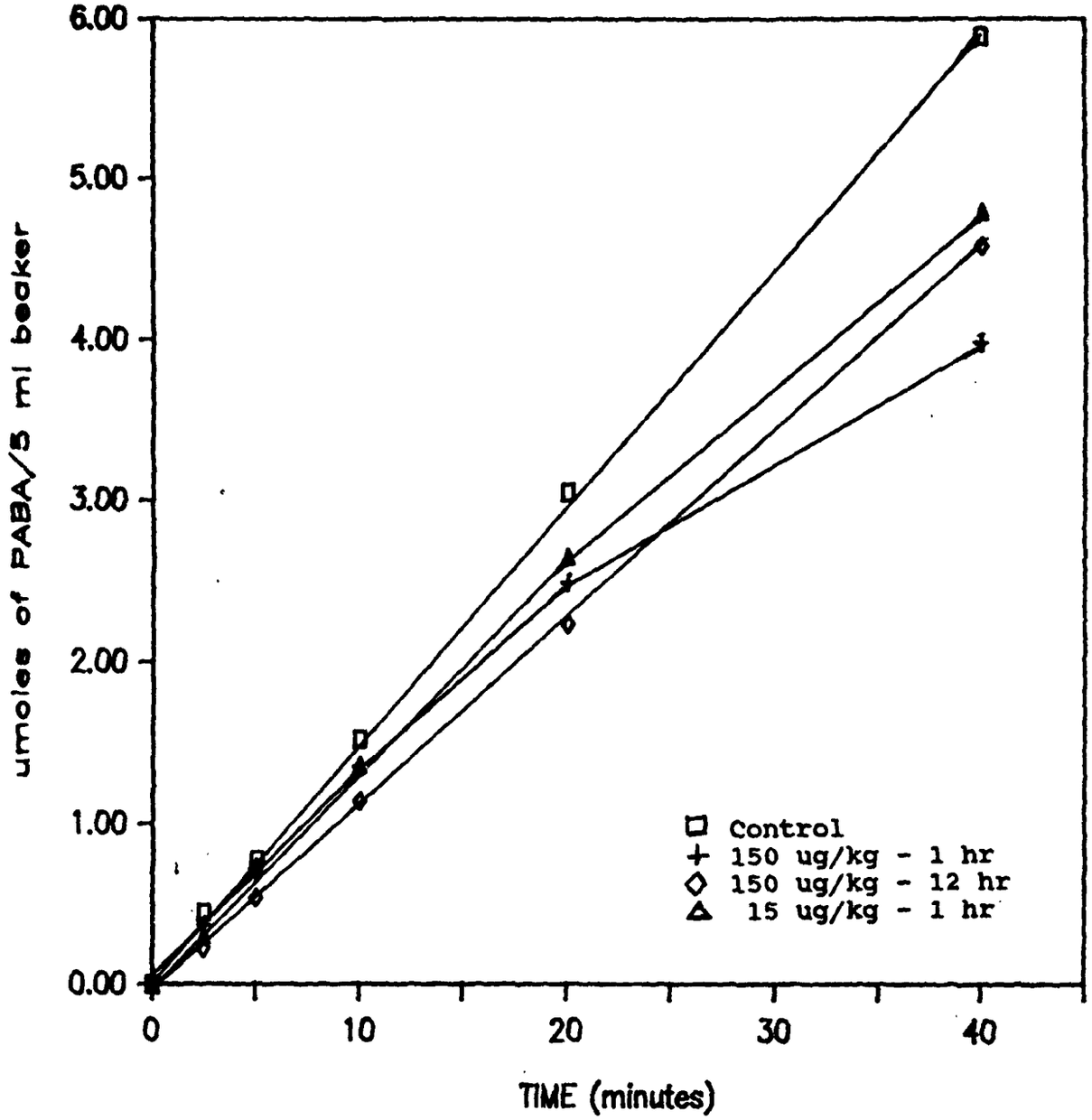

Figure 21. Linearity of the Hydrolysis of Procaine by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Procaine (1.25 mM) was incubated with 5 mg of microsomal protein.

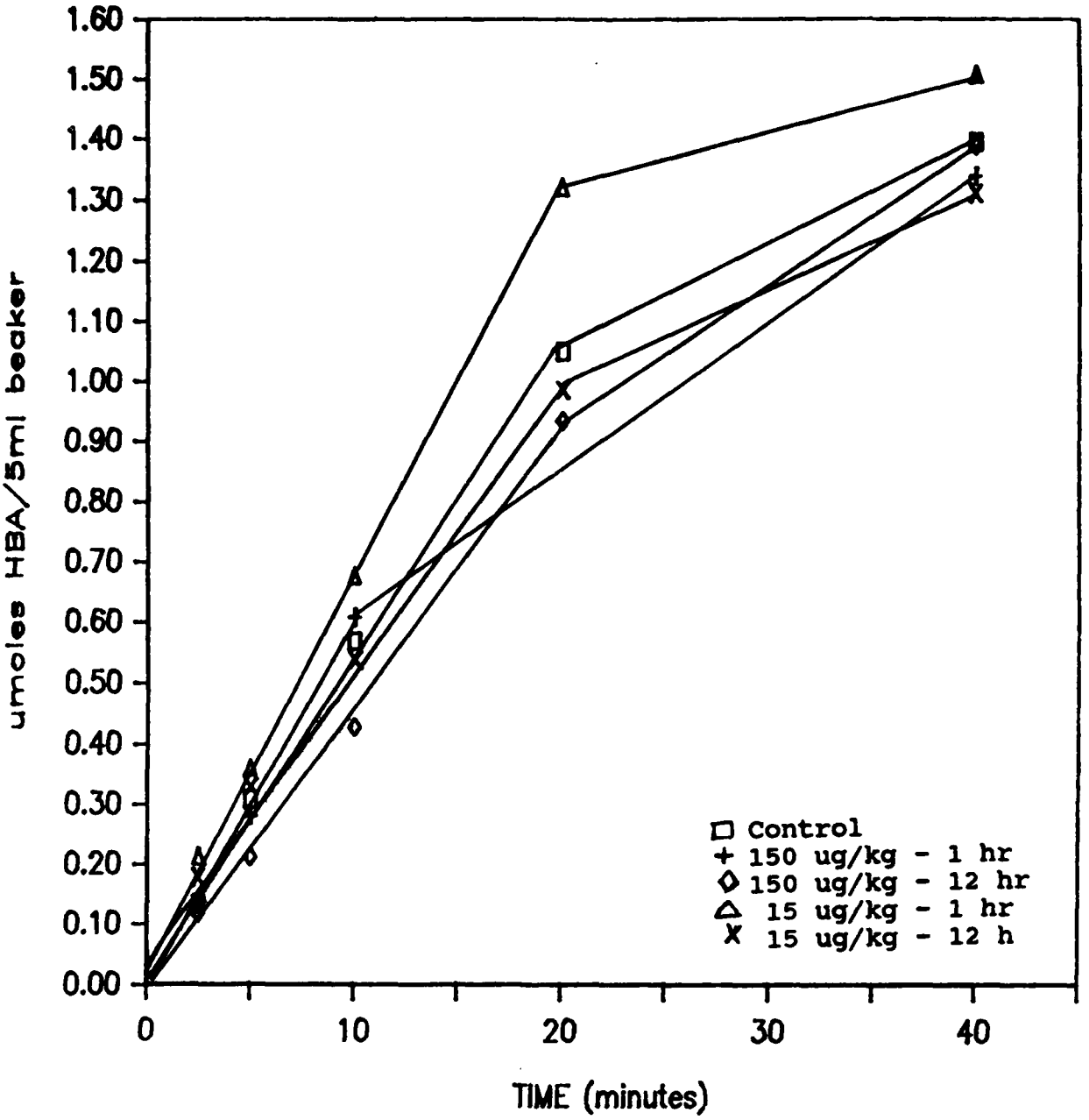

Figure 22. Linearity of the Hydrolysis of Methylparaben by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Methylparaben (0.25 mM) was incubated with 5 mg of microsomal protein.

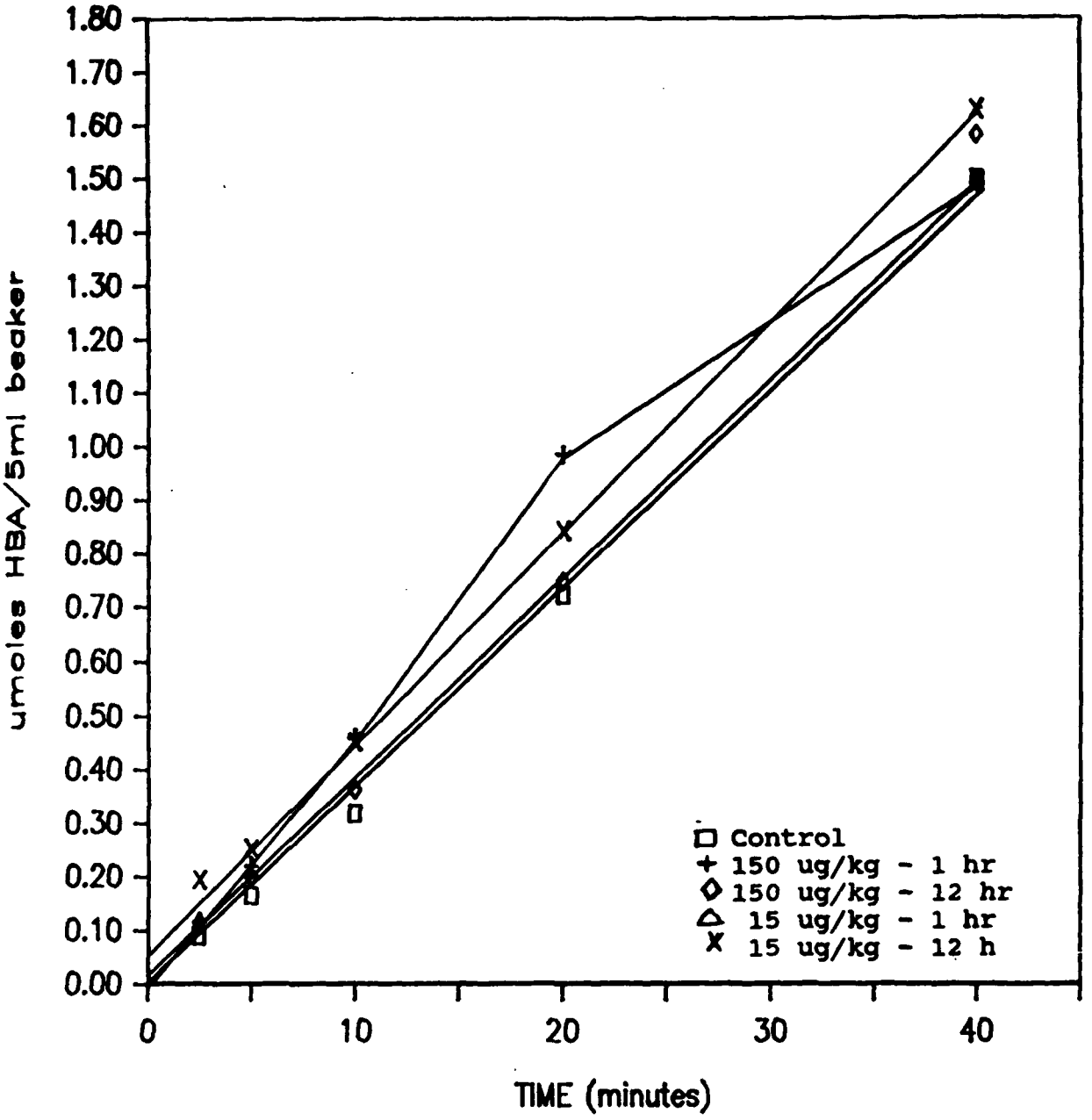

Figure 23. Linearity of the Hydrolysis of Methylparaben by Liver Microsomes from Paraoxon-Pretreated Guinea Pigs. Methylparaben (1.00 mM) was incubated with 5 mg of microsomal protein.

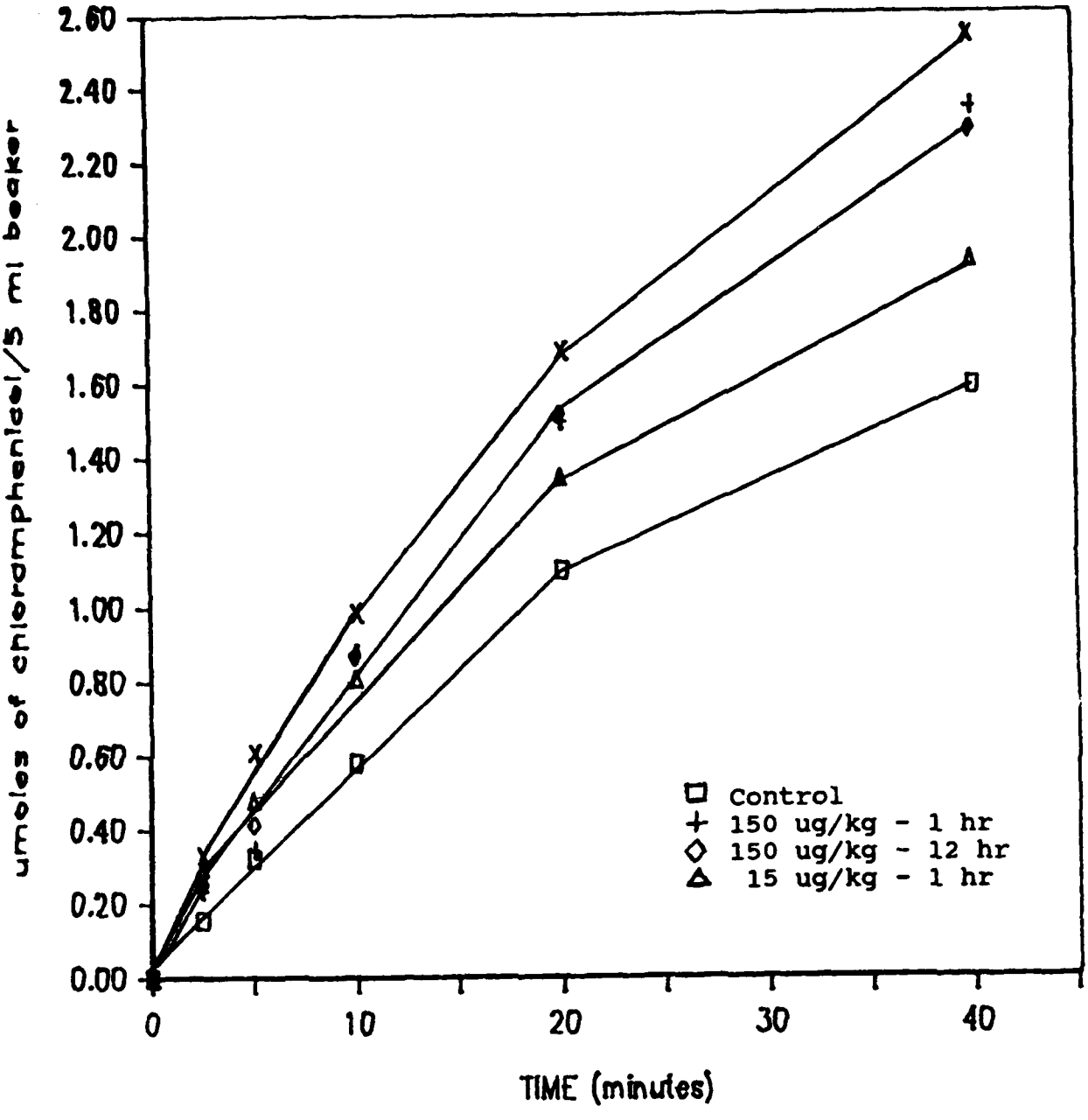

Figure 24. Linearity of the Hydrolysis of Chloramphenicol Succinate by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Chloramphenicol succinate (0.125 mM) was incubated with 5 mg of microsomal protein.

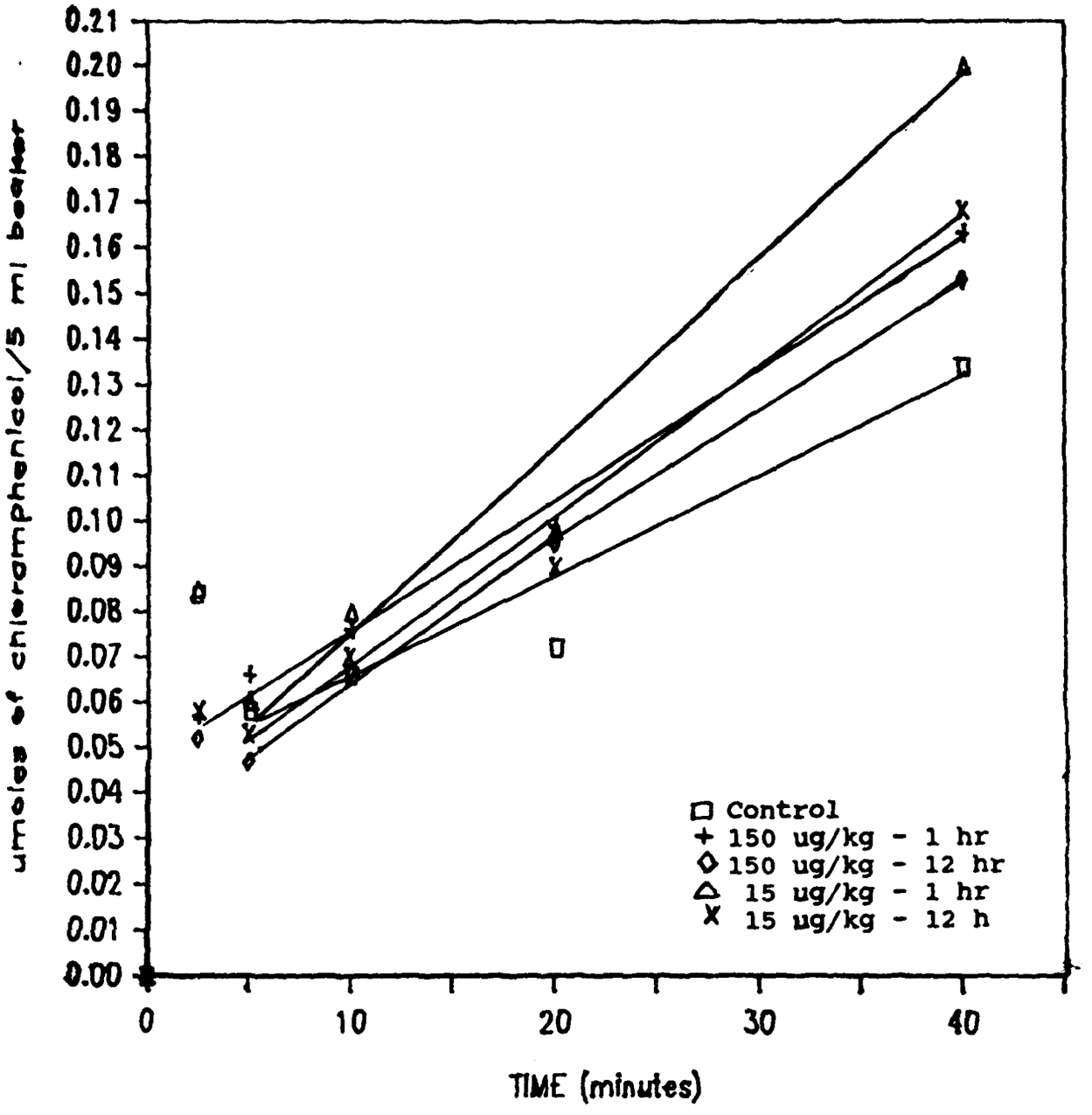

Figure 25. Linearity of the Hydrolysis of Chloramphenicol Succinate by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Chloramphenicol succinate (1.00 mM) was incubated with 5 mg of microsomal protein.

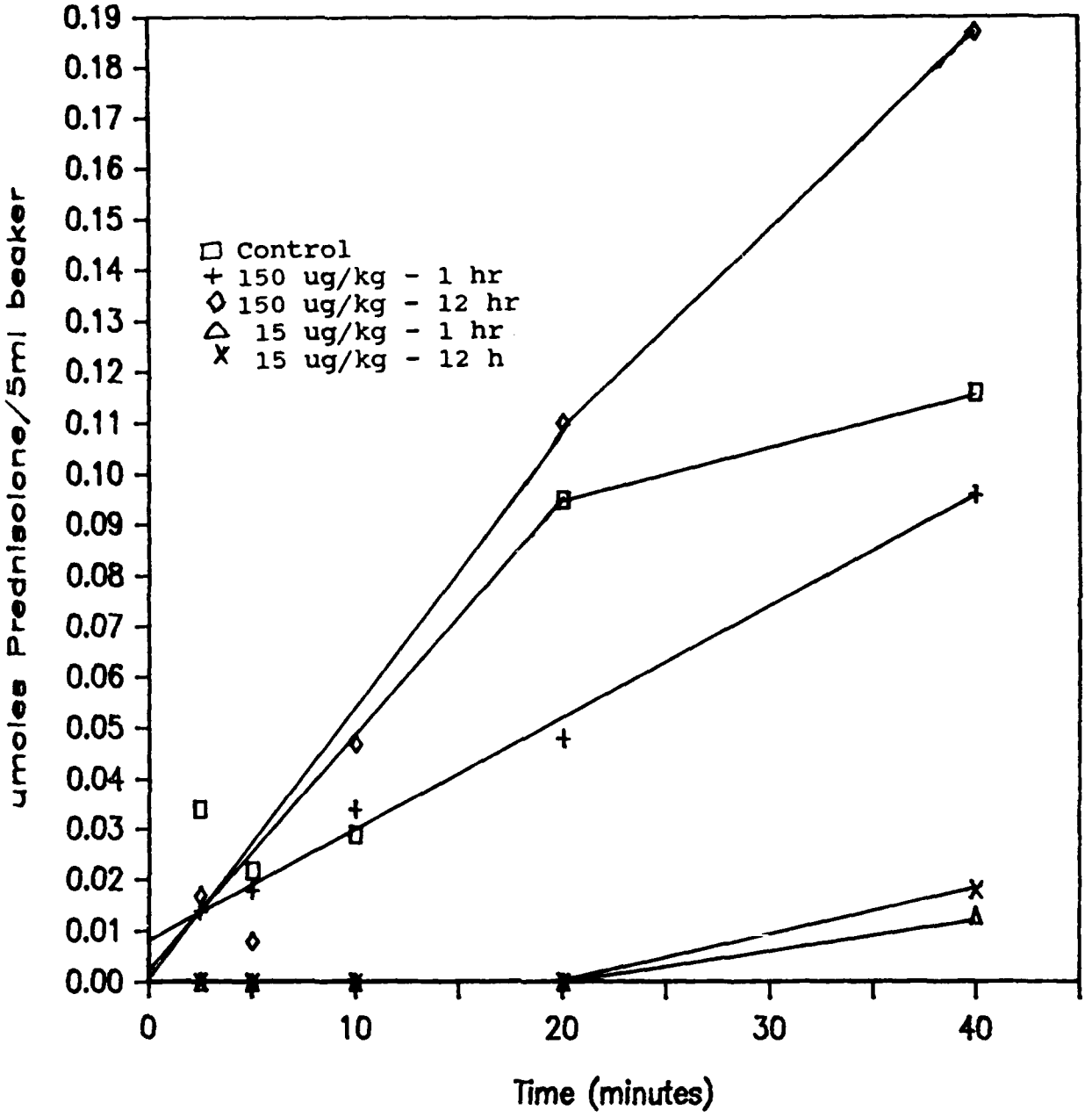

Figure 26. Linearity of the Hydrolysis of Prednisolone Succinate by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Prednisolone succinate (0.125 mM) was incubated with 5 mg of microsomal protein.

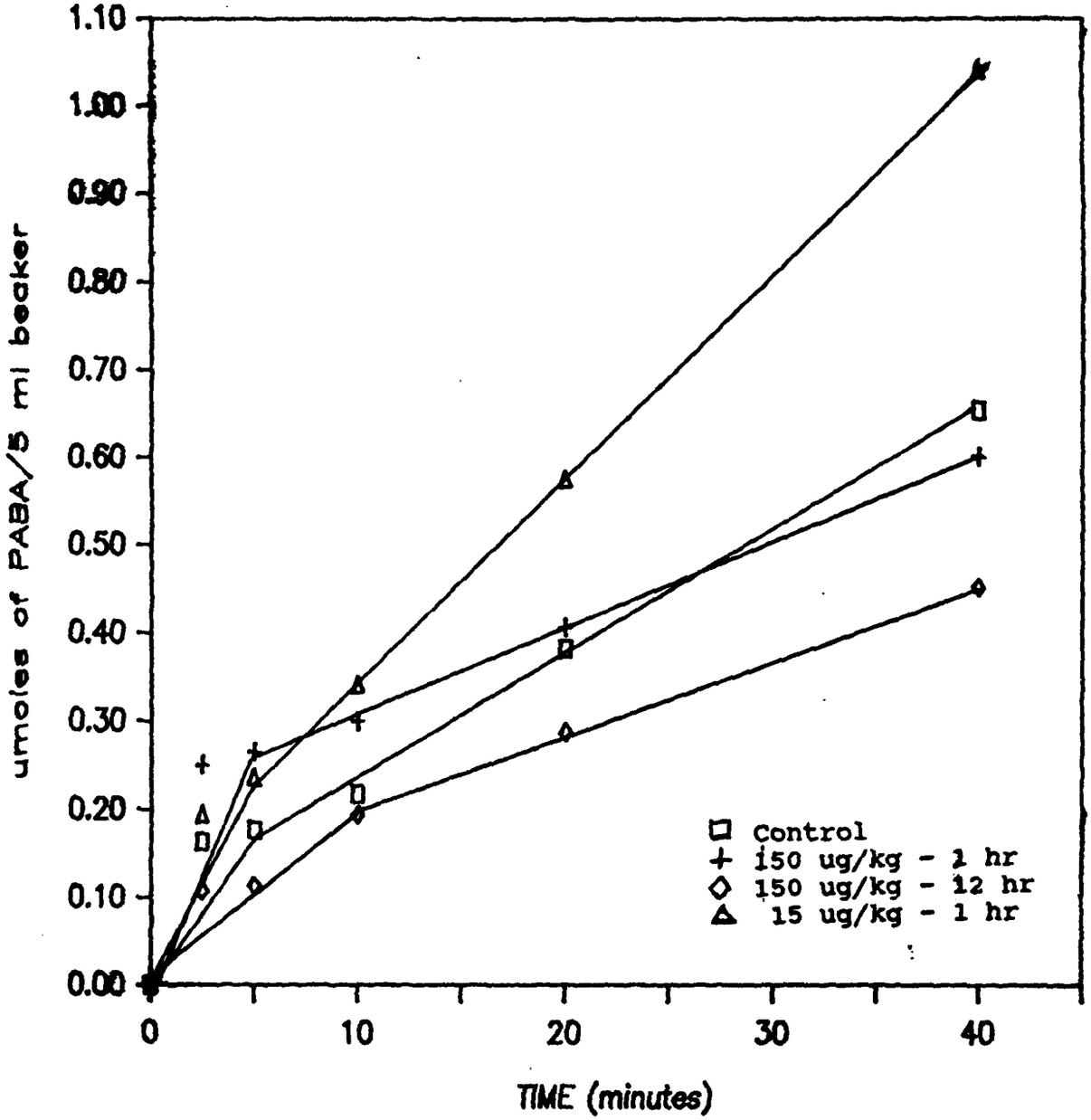

Figure 27. Linearity of the Hydrolysis of Procaine by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Procaine (0.50 mM) was incubated with 5 mg of microsomal protein.

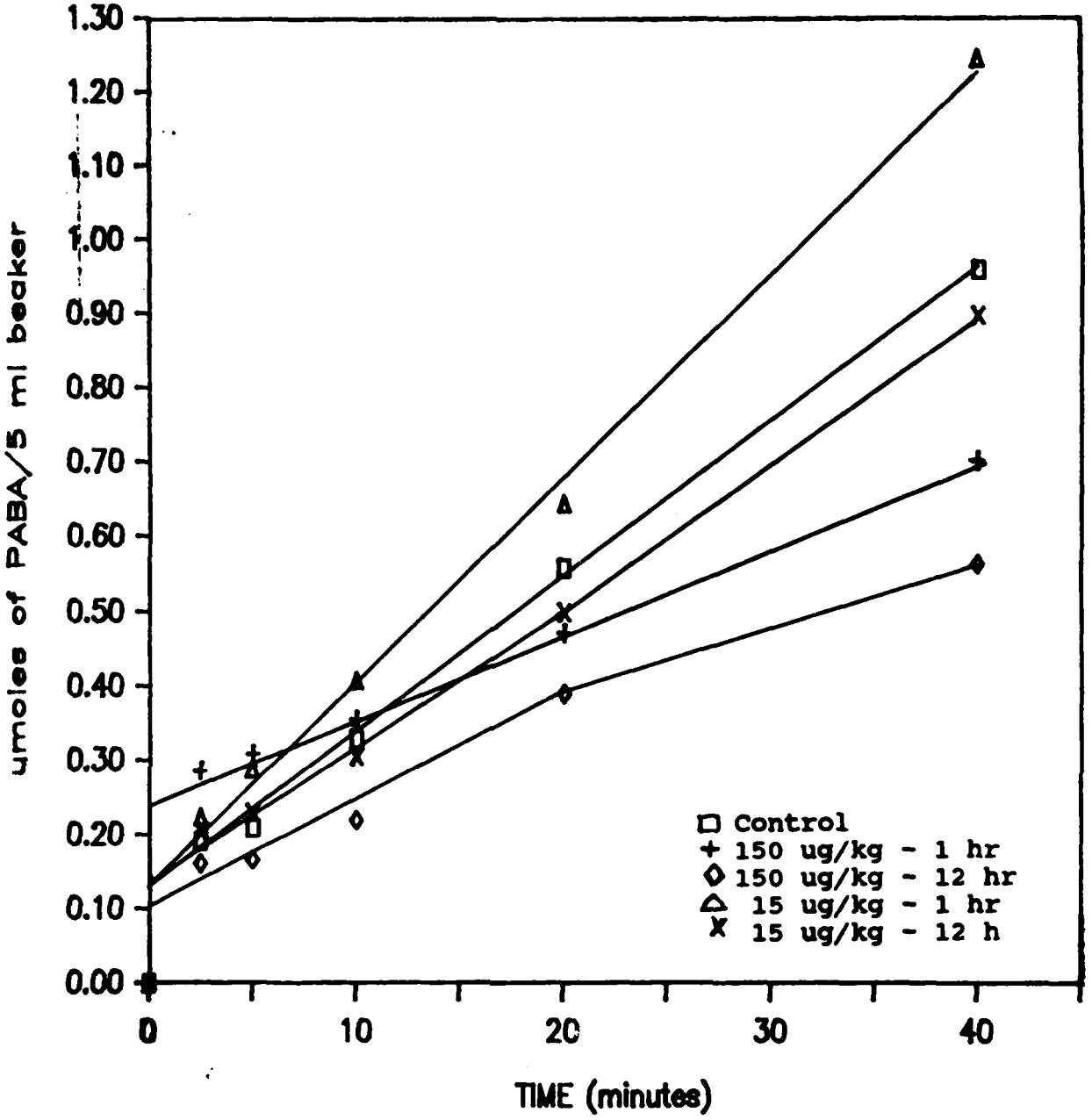

Figure 28. Linearity of the Hydrolysis of Procaine by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Procaine (1.25 mM) was incubated with 5 mg of microsomal protein.

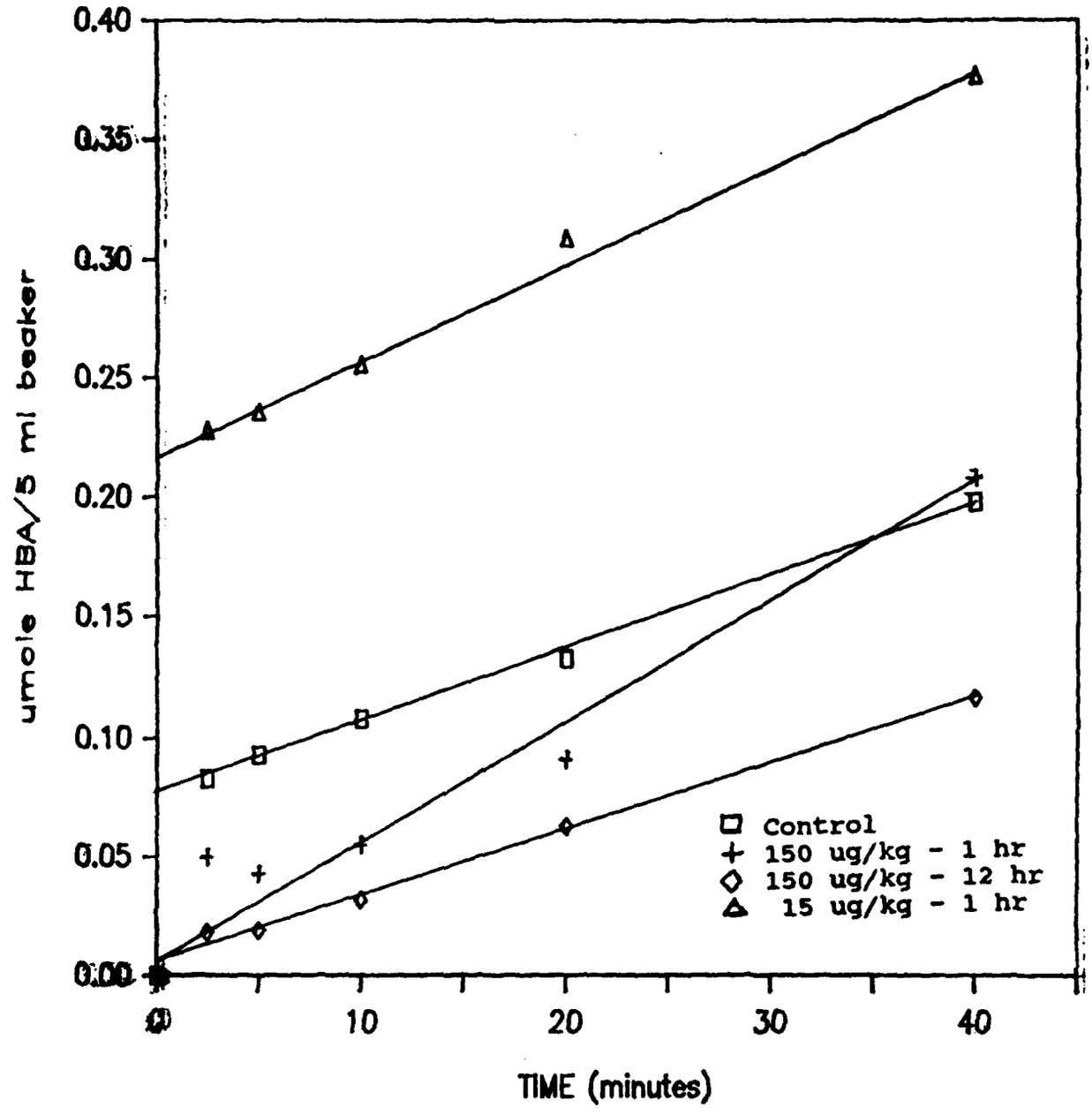

Figure 29. Linearity of the Hydrolysis of Methylparaben by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Methylparaben (0.25 mM) was incubated with 5 mg of microsomal protein.

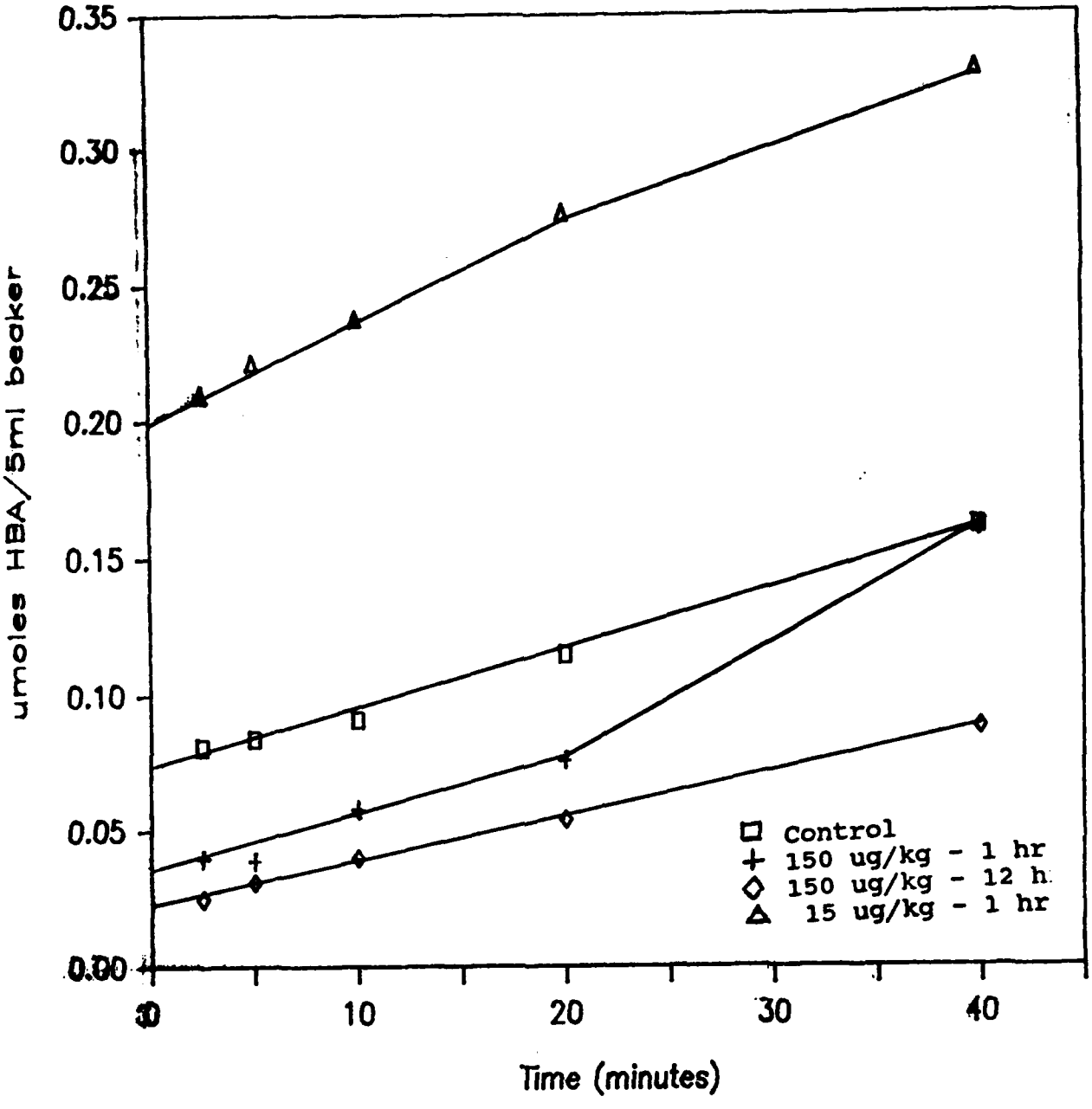

Figure 30. Linearity of the Hydrolysis of Methylparaben by Kidney Microsomes from Paraoxon-Pretreated Guinea Pigs. Methylparaben (1.00 mM) was incubated with 5 mg of microsomal protein.

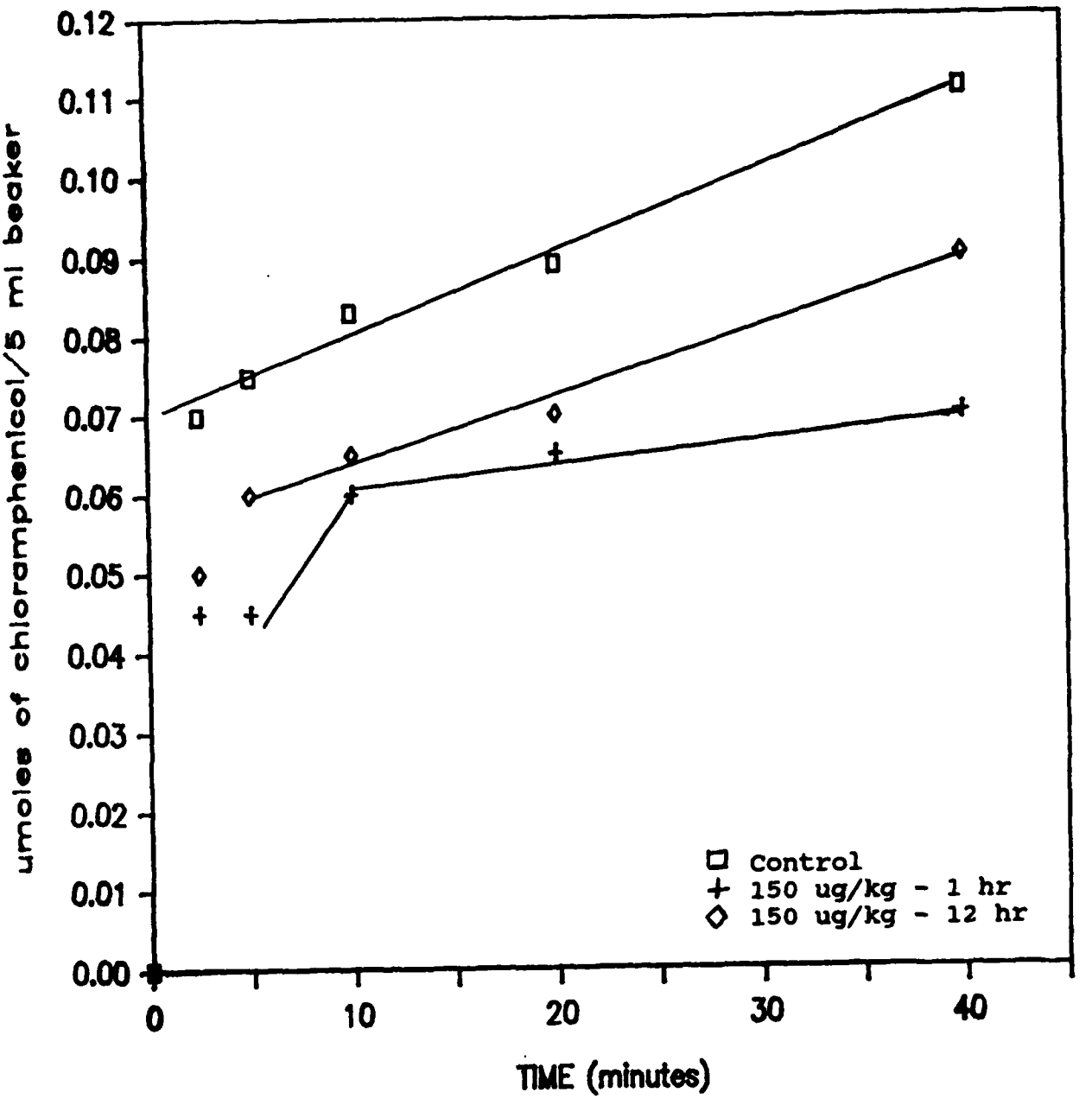

Figure 31. Linearity of the Hydrolysis of Chloramphenicol Succinate by Lung Microsomes from Paraoxon-Pretreated Guinea Pigs. Chloramphenicol succinate (0.125 mM) was incubated with 5 mg of microsomal protein.

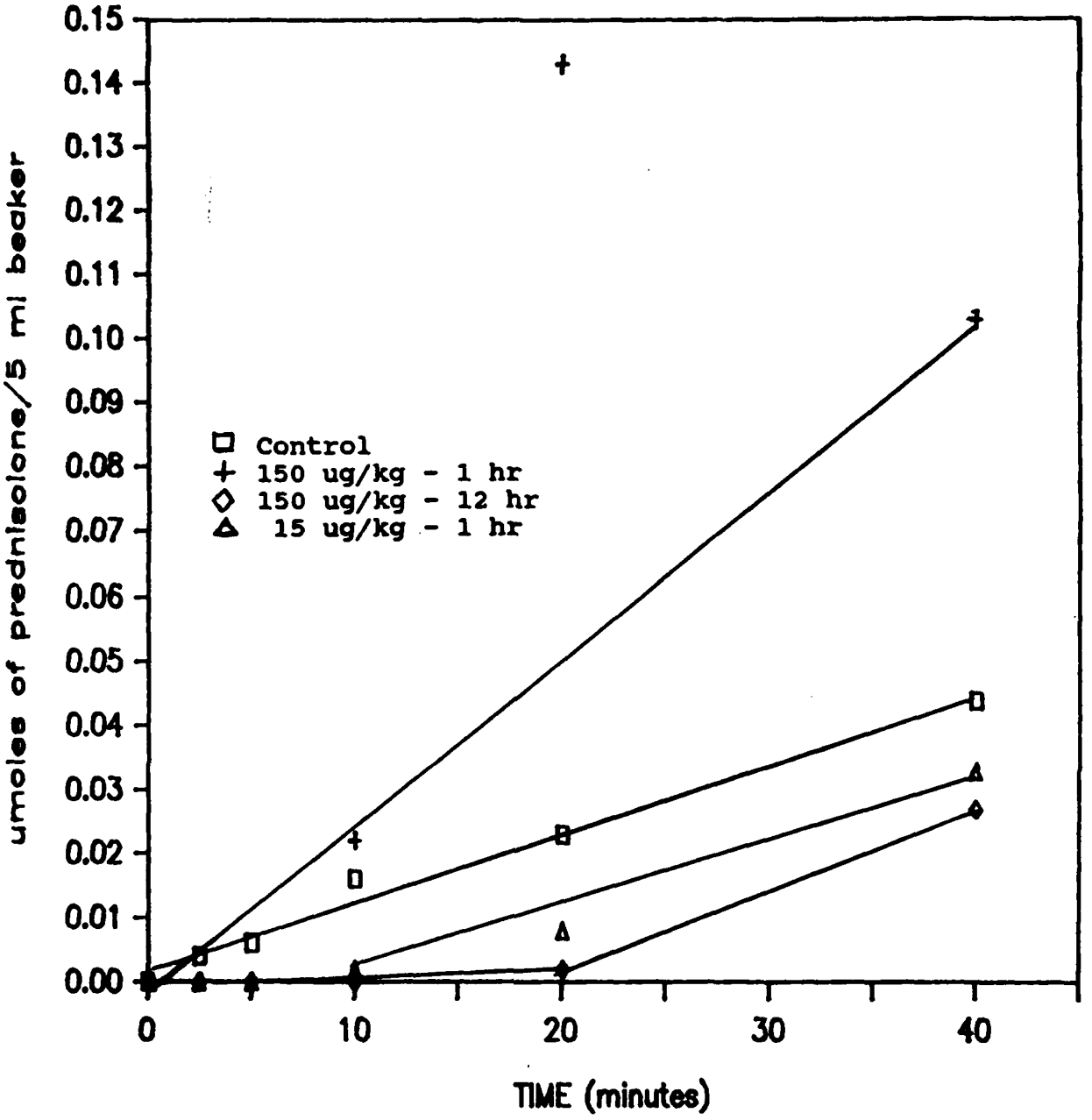

Figure 32. Linearity of the Hydrolysis of Prednisolone Succinate by Lung Microsomes from Paraoxon-Pretreated Guinea Pigs. Prednisolone succinate (0.25 mM) was incubated with 5 mg of microsomal protein.

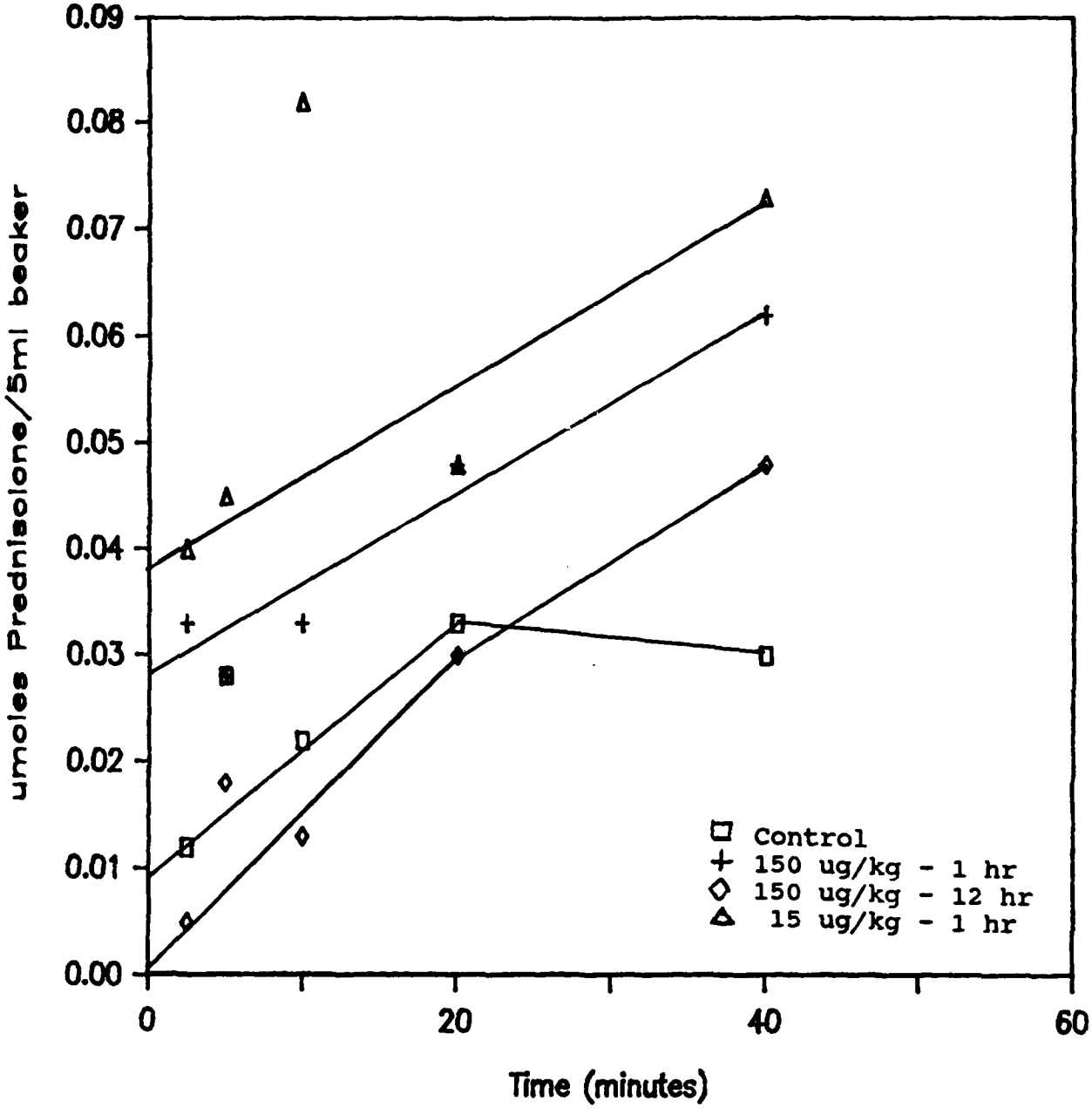

Figure 33. Linearity of the Hydrolysis of Prednisolone Succinate by Lung Microsomes from Paraoxon-Pretreated Guinea Pigs. Prednisolone succinate (1.00 mM) was incubated with 5 mg of microsomal protein.

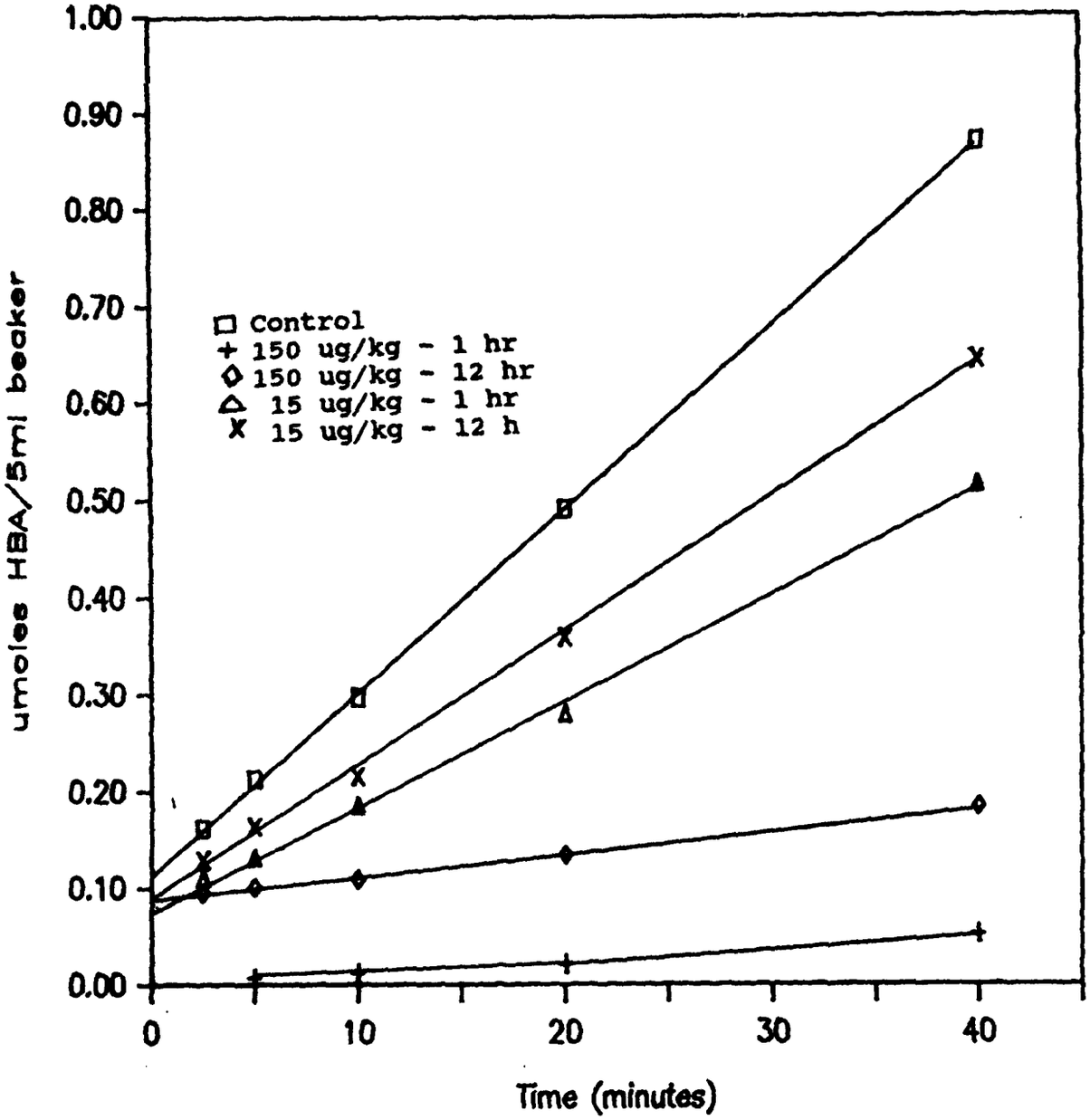

Figure 34. Linearity of the Hydrolysis of Methylparaben by Lung Microsomes from Paraoxon-Pretreated Guinea Pigs. Methylparaben (0.50 mM) was incubated with 5 mg of microsomal protein.

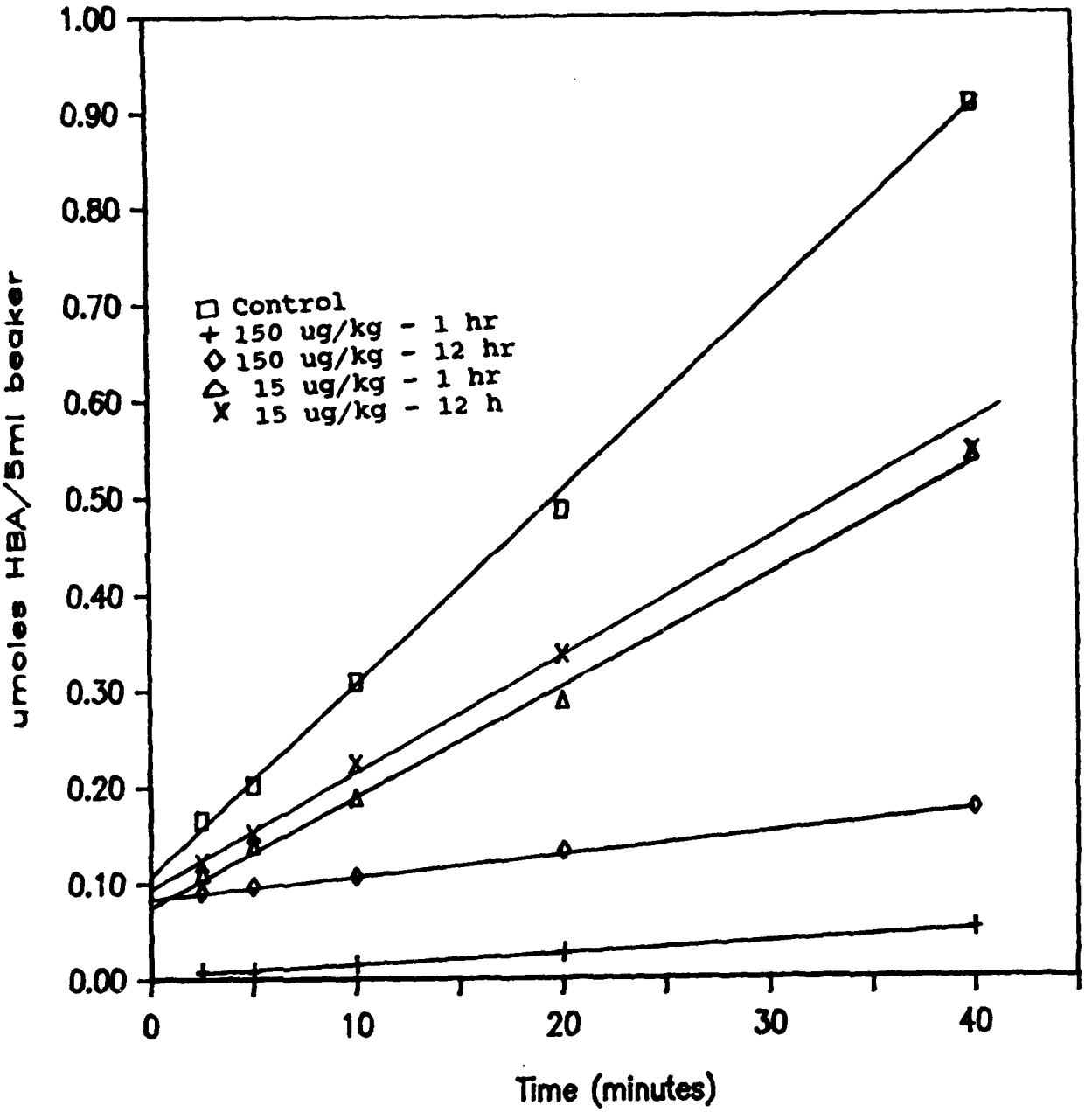

Figure 35. Linearity of the Hydrolysis of Methylparaben by Lung *Microsomes from Paraoxon-Pretreated Guinea Pigs.* Methylparaben (1.00 mM) was incubated with 5 mg of microsomal protein.

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